In vivo biotransformation of (poly)phenols and anthocyanins of red-fleshed apple and identification of intake biomarkers

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Abstract

The aim of this study was to investigate comprehensively the metabolic pathways and human bioavailability of anthocyanins and other phenolic compounds in apple matrix, and to elucidate potential intake biomarkers. After the acute intake of a red-fleshed apple snack, plasma and urine were collected and analyzed by UPLC-MS/MS. A total of 37 phase-II and microbial phenolic metabolites were detected in plasma and urine. Among these, phloretin glucuronide, cyanidin-3-*O*-galactoside (plasma and urine) and peonidin-3-*O*-galactoside (urine) were the only metabolites detected in all the volunteers and not detected at basal conditions. The maximum urine excretion was detected at 2-4 h, and the main increase in plasma of phloretin glucuronide and cyanidin-3-*O*-galactoside was observed at 2h post-intake (61.0 ± 6.82 and 10.3 ± 1.50 nM, respectively). These metabolites could be selected as the best intake biomarkers of red-fleshed apple that might be useful in human intervention studies when studying the bioactivity of red-fleshed apple.

Keywords: anthocyanins, metabolic pathways, phenolic compounds, red-fleshed apple, UPLC-MS/MS.

1. INTRODUCTION

Apples are one of the most commonly consumed fruits and their diverse and high (poly)phenol content is considered one of the most important determinants of their health-promoting properties (Hyson, 2011; Bondonno et al., 2018).

In the last few years, there has been a rapidly increasing interest in potential crops for coloring food naturally without transgenic or cysgenic programs. In order to obtain better-quality apples with added healthy properties, new genotypes of apple with red-flesh have been obtained by innovative breeding strategies through cross-breeding programs with wild red-fleshed apple varieties (with poor taste) and commercial good-flavored white-fleshed apples (Deacon, www.suttonelms.org.uk). The resulting red-fleshed apples contain a high amount of anthocyanin compounds in their flesh and have a good-tasting. Apart from anthocyanins, red-fleshed apples are also a rich source of other (poly)phenols that are also detected in common apple varieties such as phenolic acids, dihydrochalcones, flavan-3-ols, and flavonols (Bars-Cortina et al. 2017). Due to the enhanced content of anthocyanins reported in these red-fleshed apples, different studies have shown that the total phenolic content and antioxidant capacity were significantly higher in red-fleshed apples compared to traditional white-fleshed apples, which indicates that these apples could have presumably added healthy properties (Rupasinghe et al., 2010; Bars-Cortina et al. 2017).

Regarding the bioavailability of apple phenolic compounds, only a few studies have investigated the metabolism of these compounds in common varieties of apple and most of them were focused on the bioavailability after apple juice (Kahle et al.; 2011; Trošt et al. 2018) or apple cider consumption

(DuPont et al., 2002; Marks et al., 2009), with only one study reporting the phenolic metabolites after consumption of apple fruit (Saenger et al., 2017). Concerning the bioavailability of anthocyanins, there are plenty of studies reporting their human bioavailability and metabolism, however, they have been only studied in other food matrices such as blueberries, elderberries, blackcurrants, strawberries and red grapes or red wine (Wu et al., 2002; Bitsch et al., 2004; Stalmach et al., 2012; Kuntz et al., 2015; Zhong et al., 2017). So, to our knowledge, no study has been reported in the literature regarding the bioavailability of common apple phenolic compounds together with anthocyanidins in the same food matrix, which represents a specific characteristic of red-fleshed apple varieties.

In the case of anthocyanins, various types of food samples have been used to determine the effects of food matrix on their bioavailability. For instance, anthocyanins in strawberries, blood oranges and red wine have been reported to be highly bioavailable with their urinary levels varying between 1-5% of the ingested dose (Wallace et al., 2016). The differences reported in anthocyanin bioavailability from different food sources, to a large extent, is due to the presence of several structurally diverse anthocyanins in these foods, and the interactions between food matrix and these specific anthocyanins. Therefore, human postprandial studies are very useful and can contribute to knowledge about the food matrix affecting polyphenol bioavailability (Motilva et al. 2015).

Moreover, the measurement of dietary exposure and reliable intake biomarkers before investigating the potential health benefits of a new food product is of crucial importance for the discovery of unbiased associations

between the intake of bioactive compounds and the observed effects (Dragsted et al. 2018).

So, considering the scarce data regarding the human bioavailability and metabolism of apple phenolic compounds, in the present work we aimed to investigate the bioavailability and the complex metabolic pathways of the red-fleshed apple as an innovative food source rich in different polyphenols, including anthocyanins. Among all the identified metabolites, we also aimed to identify and select those plasmatic and urinary metabolites that could be considered as potential intake biomarkers of red-fleshed apple consumption and might be used to establish the relationship between their intake and health benefits in future human intervention studies.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

Cyanidin-3-*O*-galactoside, eriodictyol, quercetin-3-*O*-glucoside, quercetin-3-*O*-rhamnoside, dimer B_2, phloretin-2'-*O*-glucoside, *p*-coumaric acid, and caffeic acid were purchased from Extrasynthese (Genay, France). *p*-Hydroxybenzoic acid, 3,4-dihydroxybenzoic acid (aka protocatechuic acid), hippuric acid, 3-(4'-hydroxyphenyl)acetic acid, 3-(3',4'-dihydroxyphenyl)acetic acid, 3-(3'-hydroxyphenyl)propionic acid, 3-(3',4'-dihydroxyphenyl)propionic acid (aka dihydrocaffeic acid), 3-(3'-hydroxy-4'-methoxyphenyl)propionic acid (aka dihydroferulic acid), epicatechin, and chlorogenic acid were from Sigma-Aldrich (St. Louis, MO, USA). Vanillic acid and ferulic acid were from Fluka (Buchs, Switzerland). Vanillic acid-4-*O*-sulphate, catechol-4-*O*-sulphate, and 4-methyl

catechol sulphate were synthesized according to Pimpao et al. (2015) and were kindly supplied by Dr. Claudia N. Santos (Portugal).

Methanol (HPLC grade), acetonitrile (HPLC grade), and acetic acid were purchased from Scharlab Chemie (Sentmenat, Catalonia, Spain). The water used was Milli-Q quality (Millipore Corp, Bedford, MA, USA).

Stock solutions of standard compounds were prepared by dissolving each compound in methanol at a concentration of 1000 mg/L, and stored in a dark flask at −30 ºC.

2.2. Apple snack

The red-fleshed 'Redlove' apple variety was provided by NUFRI SAT (Mollerussa, Lleida, Spain), and planted in "La Rasa" experimental plot (La Rasa, Soria, Spain). To increase the useful life, obtaining good shelf-stability and, at the same time, minimize changes in the bioactive compounds of red-fleshed apples, the freeze-dried snack format was selected. Before drying, the apples were washed, and dried. Then, the apple core was removed and the whole apple (with peel) was cut into 1 cm-sized cubes. The apple cubes were frozen in liquid nitrogen and lyophilization was then performed with a first drying at 0.6 bar with a temperature ramp of −20 to 0 °C for 25 hours, followed by a second complete vacuum drying with a temperature ramp of 0 to 20 °C for 40 hours (Lyophilizer TELSTAR Lyobeta 15, Terrassa, Spain). The freeze-dried apple cubes were immediately transferred to airtight plastic containers and refrigerated (2 ºC) until the analysis of their phenolic composition and use in the acute intake study. A photograph of the red-fleshed apple snack is shown in Graphical Abstract.

The analysis of the phenolic composition of the apple snack was based on the previous study by Bars-Cortina et al. (2017). Prior to the chromatographic analysis of the apple (poly)phenols, a fine powder of the freeze-dried samples was obtained with the aid of an analytical mill (A11, IKA, Germany). The ingested portion of the apple snack contained a total of 196 mg of phenolic compounds. The nutritional composition and the detailed phenolic composition of the red-fleshed apple snack are presented in the **Supporting Material** in **Table S1** and **Table S2**, respectively.

2.3. Human intervention study and biological sample collection

The protocol of the study was approved by the Ethical Committee of the Human Clinical Research Unit at the Arnau Vilanova University Hospital, Lleida, Spain (Approval Number: 13/2016). Ten healthy participants (five females and five males, mean age 37.3 ± 8.4 years) with a body mass index (BMI) of 18.5–24.9 kg/m^2 were enrolled. Exclusion criteria were pregnancy or lactation, any chronic medication, any antibiotic treatment during the 4 months prior to the study, cigarette smoking, alcohol intake > 80 g/day and use of dietary supplements. After two days of a diet low in phenolic compounds, the participants were invited to eat a portion of 80 g of red-fleshed apple snack after fasting overnight. Human blood samples were obtained by venipuncture before (0 h) and after the apple snack intake at 0.5, 1, 2, 4, 6, and 24 h using 6 mL Vacutainer™ tubes (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) containing ethylenediamintetra acetic acid (EDTA) as an anticoagulant. To obtain the plasma samples, the blood tubes were centrifuged at 8784 *g* for 15 min (Hettich, Tuttlingen, Germany). Aliquots were stored at −80 ºC until the

chromatographic analysis. On the other hand, urine samples were collected 12 h before and at the interval times of 0-2, 2-4, 4-8, and 8-24 h after the apple snack intake. The total volume of each sample was measured before storing the aliquots at −80 ºC until the chromatographic analysis.

2.3.1. Plasma samples

Before the chromatographic analyses, the plasma samples were pre-treated by micro-Elution solid-phase extraction (µSPE) using OASIS HLB (2 mg, Waters, Milford, MA) micro-cartridges. The methodology used is the one reported in a previous study (Martí et al., 2010), but with some modifications. Briefly, the micro-cartridges were conditioned sequentially with 250 µL of methanol and 250 µL of 0.2% acetic acid. 350 µL of 4% phosphoric acid was added to 350 µL of the plasma sample, and then this solution was loaded into the micro-cartridges. The loaded micro-cartridges were cleaned up with 200 µL of Milli-Q water and 200 µL of 0.2% acetic acid. Then, the retained phenolic compounds were eluted with 2 x 50 µL of methanol. Each sample was prepared in triplicate.

2.3.2. Urine samples

The urine samples were also pre-treated by µSPE. The micro-cartridges and their conditioning and equilibration steps were the same as reported for plasma samples. In this case, 100 µL of phosphoric acid at 4% was added to 100 µL of the urine sample, and this solution was loaded into the micro-cartridge. The retained phenolic compounds were then eluted with 2 x 50 µL of methanol. Each sample was prepared in triplicate.

2.4. Ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS)

LC analyses were carried out on an AcQuity Ultra-Performance™ liquid chromatography and tandem mass spectrometry equipment from Waters (Milford, MA, USA). Two chromatographic methods were used for the analysis of 1) anthocyanins and their metabolites, and 2) the rest of the phenolic compounds and their metabolites. In both methods, the flow rate was 0.3 mL/min and the injection volume 2.5 µL. The UPLC.MS/MS conditions were the same used in our previous studies (Martí et al. 2010; Bars-Cortina et al. 2017; Yuste et al. 2018). Tandem MS analyses were carried out on a triple quadrupole detector (TQD) mass spectrometer (Waters, Milford, MA, USA) equipped with a Z-spray electrospray interface. The selected reaction monitoring (SRM) transition for quantification and the cone voltage and collision energy for the analysis of the phenolic metabolites are shown in **Table 1**.

Due to the lack of commercial standards of phenolic metabolites, some of these compounds were tentatively quantified by using the calibration curve of their precursor or another phenolic compound with a similar structure. Methyl catechol glucuronide was tentatively quantified by using the calibration curve of 4-methyl catechol sulphate. For hydroxybenzoic acid sulphate, the calibration curve of *p*-hydroxybenzoic acid was used; hydroxyhippuric acid with hippuric acid; protocatechuic acid sulphate with protocatechuic acid; vanillic acid glucuronide with vanillic acid; hydroxyphenylacetic acid sulphate and hydroxyphenylacetic acid glucuronide with 3-(4'-hydroxyphenyl)acetic acid; dihydroxyphenylacetic acid sulphate and dihydroxyphenylacetic acid glucuronide with 3-(3',4'-dihydroxyphenyl)acetic acid; hydroxyphenylpropionic

acid sulphate and hydroxyphenylpropionic acid glucuronide with 3-(3'-hydroxyphenyl)propionic acid; dihydroxyphenylpropionic acid sulphate with 3-(3',4'-hydroxyphenyl)propionic acid; hydroxymethoxyphenyl propionic acid sulphate (dihydroferulic acid sulphate) with 3-(3'-hydroxy-4'-methoxyphenyl)propionic acid (dihydroferulic acid); coumaric acid sulphate with *p*-coumaric acid; caffeic acid sulphate with caffeic acid; ferulic acid sulphate with ferulic acid; hydroxyphenyl-γ-valerolactone sulphate, dihydroxyphenyl-γ-valerolactone, dihydroxyphenyl-γ-valerolactone glucuronide, dihydroxyphenyl-γ-valerolactone sulphate glucuronide, epicatechin sulphate, epicatechin glucuronide, and methyl epicatechin glucuronide with epicatechin; phloretin sulphate, phloretin glucuronide and phloretin sulphate glucuronide with phloretin-2'-*O*-glucoside; cyanidin arabinoside and peonidin-3-*O*-galactoside with cyanidin-3-*O*-galactoside.

2.5. Statistical analysis

The results are presented as mean values ± standard deviation (SD) for (poly)phenols in red-fleshed apple snacks, and as mean values ± standard error of the mean (SEM) for metabolites in the urine and plasma samples. For the 8 metabolite groups (catechol/pyrogallol derivatives, benzoic acid derivatives, phenylacetic/phenylpropionic acid derivatives, phenyl-γ-valerolactone derivatives, flavan-3-ol derivatives, phloretin derivatives, and anthocyanin derivatives), one-way repeated measures analysis of variance (ANOVA) was performed on the urine samples to compare the mean differences at the five defined time points. Post hoc analysis was conducted using pairwise comparisons with Bonferroni correction. Differences were considered significant

at $p < 0.05$. All statistical analyses were performed using the SPSS v 22.0 software package.

3. RESULTS AND DISCUSSION

3.1. Red-fleshed apple snack phenolics characterization

The total amount of phenolic compounds in the apple snack portion (80 g) administered to the volunteers accounted for 196 ± 10.7 mg and the analysis of the phenolic composition showed a wide range of phenolic groups (**Table S2** Supporting Material). The more abundant phenolic compounds were phenolic acids (45%), mainly chlorogenic acid; flavan-3-ols (7%), mainly epicatechin and its dimer; the flavonols (9%), mainly quercetin derivatives, and dihydrochalcones (17%), with phloretin glucoside being the main representative and a unique compound characteristic for apples.

Different from common apple species, red-fleshed apples, have an added value as they also contain around 22% of anthocyanins. Anthocyanins in red-fleshed apple are located both in the peel and flesh (Bars-Cortina, et al., 2017), and are mainly represented by cyanidin-3-*O*-galactoside. This specific anthocyanin has only been detected in considerable amounts in chokeberry (*Aronia melanocarpa*) and lingonberry (*Vaccinium vitis-idaea*) (Zheng et al. 2003), both fruits that usually do not form part of a regular diet. Therefore, cyanidin-3-*O*-galactoside could be considered a very characteristic compound from red-fleshed apple and white-flesh red-skin apples.

Regarding the administered dose, anthocyanins have demonstrated beneficial effects at variable administered doses (7.35-640 mg/day) (Wallace, et al. 2016). Considering this range described in the literature as effective in the

prevention of chronic diseases, in the present study we selected a dose of anthocyanins around 50 mg/day (**Table S2** Supporting Material) administered through a feasible amount of apple snack (80 g/day) that could be consumed daily by the volunteers without difficulty.

3.2. Identification of the biological apple phenol metabolites by UPLC-MS/MS

In order to identify the phenolic metabolites generated after the acute intake of the red-fleshed apple snack, the detector system tandem MS was used due to its specificity, sensitivity and selectivity. The generated metabolites were determined and identified by the full scan mode in the MS mode, and in the daughter scan and SRM modes in the tandem MS mode. In addition to the detector system (MS/MS), authentic standards were also used when they were available to determine their retention time and identify the phenolic metabolites generated in plasma and urine samples. Their MS spectrum is shown in **Figure S1** in **Supporting Material**).

Although the volunteers spent two days on a diet low in phenolic compounds prior to the intervention day, some phenolic acids (phenylpropionic, phenylacetic, benzoic, and hydroxycinnamic acids) were detected and quantified in the analysis of the basal plasma and urine (fasting conditions) collected just before the apple snack intake (see **Table S3** and **S4** in **Supporting Material**). After subtraction of these basal levels of phenolics, a total of 37 phenolic metabolites were detected in the urine and/or plasma samples in increased amounts after the red-fleshed apple snack intake (**Table 1,** and **Tables S3** and **S4** in **Supporting Material**). These metabolites included

four catechol and pyrogallol derivatives, six benzoic acid derivatives, five phenylacetic acid derivatives, six phenylpropionic acid derivatives, and four hydroxycinnamic acid derivatives. Four metabolites were hydroxyphenyl-γ-valerolactone derivatives, three epicatechin derivatives, two phloretin derivatives and three cyanidin derivatives.

The phenolic metabolites were mainly phase-II sulphated (18), glucuronided (11) and methylated (8) conjugates formed through the action of the enzyme sulphotransferases (SULFs), uridine-5'-diphosphate glucuronosyltransferases (UGT), and catechol-*O*-methyltransferases (COMT), respectively. From all these phase-II metabolites, sulphation was the main transformation. The generation of simple phenolic acids, such as phenylpropionic, phenylacetic, and benzoic acid derivatives, is probably the result of microbial transformations occurring in the colon, which include ring fission, reduction, α-oxidation (one decarboxylation), β-oxidation (two decarboxylations), dehydroxylation and demethylation. In addition, phase-I metabolism (dehydrogenation or reduction) may also be involved in the formation of these metabolites. These simple phenolic acids can then undergo phase-II metabolism at the colon level and/or be absorbed and reach the liver, where they would be subject to enzymatic metabolism before re-entering the systemic blood circulation and finally being excreted in the urine.

3.3. Proposed metabolic pathways of red-fleshed apple phenols

Based on the diversity of phenolic metabolites, whose concentration in plasma or urine increased after the apple snack intake (**Table 1**), a complex picture of the metabolic pathways of the main apple phenolic compounds, as

well as their interactions, has been proposed. **Figure 1** shows the proposed metabolic routes to explain the phenolic metabolites generated from chlorogenic acid (in green), vanillic acid hexoside (in blue), cyanidin-3-*O*-galactoside (in orange), epicatechin and dimer B_2 (in lilac), quercetin derivatives (in pink), and phloretin (xylosyl) glucoside (in brown) determined in the red-fleshed apple snacks as the main phenolics. The name of the phase-II enzymes is shown in green, and the colonic catabolism in brown. In the next subsections, the metabolic pathways of the metabolites generated from the apple (poly)phenols are described.

3.3.1. Anthocyanins

Cyanidin-3-*O*-galactoside, and cyanidin arabinoside (but at lower concentration levels), were the main anthocyanins present in the red-fleshed apple snack (Supporting Material **Table S2**). These cyanidin glycosides (galactoside and arabinoside) were also detected in both the plasma and urine samples in their native structure detected in apple snack. Other anthocyanin metabolites were also identified in the plasma and urine, derived from phase-II metabolism and microbial metabolism (**Figure 1).** Regarding phase-II metabolites, peonidin-3-*O*-galactoside was detected in the urine resulting from cyanidin-3-*O*-galactoside methylation by the action of COMT enzyme. Methylation, as one of the first metabolic reactions of cyanidin glycosides, was also reported by other authors in plasma and urine samples after the acute intake of aronia berry extract (Xie et al. 2016), and also after the oral ingestion of 500 mg of ^{13}C-labelled cyanidin glucoside (De Ferrars et al. 2014).

Other cyanidin metabolites, based on the B-ring fission and cleavage of the C-ring by the action of colonic enzymes (Mosele et al. 2015), were also detected in our study. As a result, protocatechuic acid and dihydroxyphenylpropionic acid (dihydrocaffeic acid) were respectively detected. Protocatechuic acid might also have been formed by β-oxidation of dihydroxyphenylpropionic acid. Then, as proposed in **Figure 1** (orange arrows), protocatechuic acid could either be further degraded by the action of the microbial flora to catechol metabolites (α-oxidation), pyrogallol metabolites (hydroxylation) and hydroxybenzoic acid (dehydroxylation), or methylated to vanillic acid.

Despite B-ring fission and C-ring cleavage, phloroglucinol sulphate could have been generated from A-ring fission. Nevertheless, this metabolite could not be differentiated from pyrogallol sulphate due to the lack of commercially available standards and because these two metabolites have the same precursor (*m/z* 205) and product ions (*m/z* 125 and 83) (See **Table 1**). Therefore, this metabolite could be tentatively identified as phloroglucinol sulphate due to the A-ring fission (metabolic pathways not shown), or as pyrogallol sulphate due to hydroxylation of catechol.

3.3.2. Other phenolic compounds

Chlorogenic acid (see **Figure 1** green arrows). Caffeic acid would be the first metabolite generated from chlorogenic acid by ester hydrolysis (**Figure 1**). From this metabolite (caffeic acid), different reactions based on microbial metabolism (dehydroxylation), and phase-I (dehydrogenation or reduction) and phase-II (COMT) metabolism could occur resulting in the generation of coumaric acid, dihydroxyphenylpropionic acid and ferulic acid, respectively.

Then, dihydroxyphenylpropionic acid and ferulic acid could be further degraded to phenylpropionic acid, phenylacetic acid and vanillic acid. These metabolites could be further degraded to such simpler phenolic compounds as protocatechuic acid, *p*-hydroxybenzoic acid and catechol metabolites.

Vanillic acid hexoside (see **Figure 1** blue arrows). Vanillic acid hexoside was the second most abundant phenolic acid quantified in the red-fleshed apple snack (Supporting Material **Table S2**). After deglycosylation of this phenolic acid, vanillic acid could be formed and subsequently sulphated (vanillic acid sulphate), glucuronided (vanillic acid glucuronide) and demethylated (protocatechuic acid). Then, as has been commented before, protocatechuic acid could also be further degraded by microbial activity to generate catechol and pyrogallol metabolites.

The presence of the metabolites derived from these two phenolic acids (chlorogenic and vanillic acids) was in agreement with the results reported in the literature for the bioavailability study after the consumption of foods rich in these phenolics, such as coffee (Monteiro et al. 2007; Renouf et al 2010; Ludwig et al 2013;), cereals (Calani et al. 2014), olive oil enriched with thyme phenols (Rubió et al. 2014) and apple juice (Kahle et al. 2011).

Flavan-3-ols (epicatechin and dimer B_2) (see **Figure 1** lilac arrows). Flavan-3-ol metabolites included both phase-II and microbial catabolites. The first metabolic step would be the hydrolysis of the proanthocyanidin dimer to catechin and epicatechin, and these monomers were found in urine as glucuronided ((epi)catechin glucuronide), sulphated ((epi)catechin sulphate),

and further methylated (methyl (epi)catechin sulphate) conjugates. On the other hand, the flavan-3-ols monomers could also be metabolized by the gut microbiota to dihydroxyphenyl-γ-valerolactone, detected in urine samples. Similarly, other studies reported the valerolactones as specific flavan-3-ols metabolites (Aura, 2008; Hackman et al. 2008; Serra et al. 2011; Mosele et al. 2015). Then, dihydroxyphenyl-γ-valerolactone could also be glucuronided, and further sulphated and also dehydroxylated and further sulphated.

Quercetin derivatives (see **Figure 1** pink arrows). Quercetin galactoside/glucoside and arabinoside would firstly be deglycosylated, and then the generated aglycone (quercetin) could enter epithelial cells by passive diffusion and be absorbed. Nevertheless, in the present study, no phase-II metabolites of quercetin were identified. On the other hand, quercetin rhamnoside has been reported not to be metabolized in the small intestine and to reach the colon where this is metabolized to dihydroxyphenylpropionic acid (Arts et al. 2004; Aura, 2008; Serra et al. 2012; Mosele et al. 2015), and then progressively metabolized to generate simple phenols, such as phenylacetic and benzoic acids, down to catechol derivatives. In our study, only microbial metabolites from quercetin derivatives were observed.

Phloretin (xylosyl) glucoside (see **Figure 1** brown arrows). Phloretin (xylosyl) glucoside could be firstly deglycosylated to generate phloretin. Then, this dihydrochalcone could be glucuronided by UGT enzymes and further sulphated with SULF enzymes. Regarding the literature, phloretin glucuronide was also reported in plasma and urine samples after the oral consumption of

apple cider (Marks et al. 2009) and apple fruit (Saenger et al. 2017). This compound could also be metabolized by the gut microbiota enzymes to dihydroxyphenylpropionic acid and further metabolized to generate simpler phenolic compounds, such as phenylacetic and benzoic acids down to catechol derivatives.

3.4. Biomarkers for apple phenol consumption

As evidenced in the metabolic pathways proposed in **Figure 1**, a large number of metabolites are generated from the five main phenolic groups (anthocyanins, phenolic acids, flavan-3-ols, flavonols and dihydrochalcones) present in the red-fleshed apple snack. Some of the metabolites identified in the plasma and urine samples after the snack intake are common to several phenolic groups. These compounds are mainly colonic metabolites, such as phenylpropionic, phenylacetic, and benzoic acids, and catechol derivatives. Additionally, these phenolic metabolites are common to other (poly)phenol-rich foods. That is why, more attention was paid in this study to specific phenolic compounds detected in plasma or urine that could be used as biomarkers for red-fleshed apple intake. The identification of the specific food intake biomarkers is of great importance to establish the relationship between (poly)phenols intake and health benefits in human intervention studies.

The 37 phenol metabolites detected in the urine and plasma samples after the red-fleshed apple snack intake (see **Table 1**, **and Tables S3 and S4** in **Supporting Material)** could be classified into two groups according to their urine excretion (µmol) kinetic, as is shown in **Figure 2**. The first group would include derivatives from phenylpropionic and phenylacetic acids, benzoic acids,

catechol and pyrogallol and hydroxycinnamic acids (see **Figure 2A**); and the second group the derivatives from flavan-3-ols, valerolactones, dihydrochalcones, and anthocyanins (see **Figure 2B**). The phenolic metabolites from the first group are excreted at high concentration levels (µmol) in the different interval times (0 to 24 h). However, these phenolic compounds presented low specificity to be considered as intake biomarkers, as they were also quantified under basal conditions (before the apple intake), and only a slight but not significant increase in phenylpropionic/phenylacetic acids, benzoic acids and catechol/pyrogallol derivatives was observed after the apple intake in the urine excretion between 0 to 24 h. This fact was also observed in the plasma samples and their concentration was also only slightly enhanced after the intake of the red-fleshed apple snack (see Supporting Material **Table S3**). So, these compounds could be considered "endogenous" phenolic metabolites from the diet.

On the other hand, the phenolic metabolites from the second group were excreted in urine at concentration levels (µmol) and significantly increased (p<0.05) after the red-fleshed apple intake at the different interval times studied (see **Figure 2B**). Phenolic metabolites from this group could be considered as potential biomarkers for red-fleshed apple consumption, since they were detected at trace levels or not detected in basal conditions.

Figure 3 shows the individual profile of the urinary excretion (µmol and nmols for anthocyanins) of the potential biomarkers quantified after the red-fleshed apple snack intake. These urinary biomarkers include cyanidin galactoside (A1), cyanidin arabinoside (A2) and peonidin galactoside (A3) as cyanidin derivatives (anthocyanins); phloretin glucuronide (B1), and phloretin

sulphate glucuronide (B2) as phloretin derivatives from dihydrochalcones pathways, epicatechin sulphate (C1), methyl epicatechin sulphate (C2) and epicatechin glucuronide (C3) as epicatechin derivatives, and dihydroxyphenyl-γ-valerolactone (D1), dihydroxyphenyl-γ-valerolactone sulphate glucuronide (D2), dihydroxyphenyl-γ-valerolactone glucuronide (D3) and hydroxyphenyl-γ-valerolactone sulphate (D4) as phenyl-γ-valerolactone derivatives from flavan-3-ols pathways.

As shown in **Figure 3** and in **Table S3 (Supporting Material)**, cyanidin (A1-A3), phloretin (B1 and B2) and epicatechin (C1-C3) derivatives were excreted in the first hours after the apple intake, their maximum excretion being detected from 2 to 4 h. Then, their urinary excretion decreased until 24 h. These results are in agreement with other studies after apple consumption (DuPont et al. 2002; Mennen et al. 2006; Kahle et al. 2007; Marks et al. 2009; Kristensen et al. 2012; Saenger et al. 2017).

Note that the native forms of some apple anthocyanins, such as cyanidin-3-*O*-galactoside, cyanidin arabinoside, and peonidin-3-*O*-galactoside and also phloretin glucuronide (a phase-II metabolite) were detected in the urine samples from all the volunteers. Additionally, cyanidin-3-*O*-galactoside and phloretin glucuronide were also detected in all the plasma samples. Observing the plasma kinetic profile of these metabolites, they were rapidly absorbed in the small intestine showing a maximum concentration at 2-3 h. After that, their concentration decreased significantly until 24 h (see **Figure 4** and **Supporting Material Table S4**).

Regarding phenyl-γ-valerolactone derivatives (**Figure 3**, D1-D4) from flavan-3-ols metabolic pathways, their urinary excretion increased after the apple

snack intake showing a maximum excretion from 8 to 24 h. This trend indicates intense colonic microbial metabolism, which could explain that these compounds were not detected in the plasma. Nevertheless, in our previous study in which we analyzed whole blood by sampling with dried blood spot (DBS) cards from the same volunteers, hydroxyphenyl-γ-valerolactone glucuronide was detected after 12 h (Yuste et al. 2018). These differences could indicate higher sensitivity in the detection of circulating valerolactones from the analysis of whole blood instead of plasma.

From the results obtained in the present study, all the phenolic metabolites shown in **Figure 3** could be proposed as urinary markers for red-fleshed apple consumption. Nevertheless, epicatechin and phenyl-γ-valerolactone derivatives were not considered in the present study as biomarkers since these compounds are also present in other flavan-3-ol-rich foods, such as cocoa, wine and tea, which form part of a regular diet. Epicatechin phase-II metabolites, such as the sulphated, glucuronided and methylated derivatives found in the present study, as well as the microbial derived phenyl-γ-valerolactone metabolites, are certainly good biomarkers for the correct assessment of intake and health effects exerted by flavan-3-ol-rich diets (Van der Hooft et al. 2012; Urpi-Sardá et al. 2015). However, when proposing intake biomarkers, more specific metabolites must be sought.

On the one hand, as reported in previous studies (Mennen et al., 2006; Saenger et al., 2017) we corroborate that phloretin derived metabolites, in this study phloretin glucuronide as a phase-II metabolite was determined, are good biomarkers for apple intake, as phloretin glucoside is an exclusively apple dihydrochalcone (Richling, 2012). Moreover, phloretin glucuronide was

considered to be a good biomarkers as it was detected in all the plasma and urine samples from all the volunteers and was not detected under basal conditions.

On the other hand, cyanidin-3-*O*-galactoside, was considered also a good intake biomarker as it is the main anthocyanin of red-fleshed apples (Guo et al., 2016; Bars-Cortina et al., 2017), and apart from these apple varieties, it can be only found in considerable amounts in chokeberry and lingonberry fruits. Therefore, cyanidin-3-*O*-galactoside could be considered as good intake biomarker for red-fleshed apple, and probably also for apples with white flesh and red skin, although no data where found in the literature. This metabolite was also detected in all the plasma and urine samples from all the volunteers and was not detected under basal conditions. Peonidin-3-*O*-galactoside was also quantified in the urine samples from all the volunteers, but at lower amounts than cyanidin-3-*O*-galactoside. This methyl conjugate of cyanidin-3-*O*-galactoside (peonidin-3-*O*-galactoside) could also be selected as a good urinary biomarker for red-fleshed apple consumption jointly with phloretin glucuronide and cyanidin-3-*O*-galactoside (**Figure 4).**

As pointed out, these three phenolic metabolites (phloretin glucuronide, cyanidin-3-*O*-galactoside and peonidin-3-*O*-galactoside) are good intake biomarkers although not strictly specific for red-fleshed apples. However, the presence of both phloretin glucuronide and cyanidin galactoside metabolites (cyanidin-3-*O*-galactoside and peonidin-3-*O*-galactoside) could be considered as more specific intake biomarkers from apples with red-flesh.

Further, it is important to remark, as we previously reported (Bars-Cortina et al., 2017), that the red-fleshed apples contains a higher concentration of

anthocyanins but a lower concentration of flavan-3-ols in its flesh in comparison to the traditional white-fleshed varieties with red skin. This fact could be explained by a competitive synthesis between anthocyanins and proanthocyanidins in the flesh. Presumably this different ratio of anthocyanins/flavan-3-ols reported in red-flesh apples, might be reflected in the concentrations of the generated metabolites. So, in quantitative terms, after a red-fleshed apples intake a lower amount of flavan-3-ols metabolites would be expected in comparison to white-fleshed apples with red skin intake. To support this hypothesis, the generated metabolites after an intake of red-fleshed and traditional white-fleshed snack apples are being analyzed in an ongoing study.

Conclusions

In the present study, the different phenolic metabolites generated after the intake of red-fleshed apple snacks were identified and tentatively quantified in urine and plasma samples at different time intervals. Moreover, the metabolic pathways of the phenolic metabolites generated from red-fleshed apple phenolics were proposed, and these routes were based on phase-II and microbial reactions. The results show that after the consumption of red-fleshed apple snacks, (poly)phenols are extensively metabolized, resulting in the production of a large number of compounds with different structure, all of which should be considered when investigating the potential health effects of red-fleshed apples. Among all the metabolites generated, phloretin glucuronide, cyanidin-3-*O*-galactoside and peonidin-3-*O*-galactoside were proposed as the best candidates as biomarkers after the intake of red-fleshed apple snack. These two phenolic metabolites were not detected in basal samples and were

detected in the urine and/or plasma samples from all the volunteers. It is important to highlight that these three phenolic metabolites are not strictly exclusive to red-fleshed apple intake, since phloretin glucuronide is a common biomarker for all apple fruits, and cyanidin-3-*O*-galactoside and peonidin-3-*O*-galactoside could appear not only after the intake of red-flesh apple but also after white-flesh with red-skin apples or other fruits such as chokeberry and lingonberry. However, the presence of these three metabolites could be useful as intake biomarkers in human intervention studies when studying the bioactivity of red-fleshed apple.

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CONFLICT OF INTEREST

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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Figure captions

Figure 1. Proposed metabolic pathways for the generation of phenolic metabolites after the acute intake of red-fleshed apple snack. The metabolic route for chlorogenic acid is in green, for vanillic acid hexoside in blue, for anthocyanins in orange, from flavan-3-ols in lilac, from quercetin derivatives in pink and from dihydrochalcones in brown.

Reactions: *dH*, dehydrogenation; SULT, sulphotransferase; *UGT*, glucuronosyl-transferase; and *COMT*, catechol-O-methyltransferase; *dOH*, dehydroxylation; *dMe*, demethylation; *α-oxidation*, one decarboxylation; and *β-oxidation*, two decarboxylations.

Quercetin derivatives: quercetin glucoside, quercetin galactoside, quercetin arabinoside and quercetin rhamnoside.

Figure 2. Phenolic metabolite excretion rate in urine A) phenylpropionic/ phenylacetic acids, benzoic acids, catechol/pyrogallol and hydroxycinnamic acids derivatives; and B) flavan-3-ols, phenyl-γ-valerolactones, dihydrochalcones, and anthocyanins derivatives. Except for anthocyanin derivatives (nmol/h), data expressed as µmol/h as mean values ± standard error of mean (n=10). Asterisks indicate significant differences ($p<0.05$) in excretion rate compared to basal conditions.

Figure 3. Urinary excretion of the proposed biomarkers for red-fleshed apple consumption 0-24 h after acute intake of 80 g apple snack. A1-A3: Cyanidin derivatives; B1-B2: Phloretin derivatives; C1-C3: Epicatechin derivatives; D1-D4: Phenyl-γ-valerolactone derivatives. Except for anthocyanin derivatives

(nmol/h), data expressed as μmol/h as mean values ± standard error of mean (*n*=10).

Figure 4. Pharmacokinetic profile of the proposed biomarkers for red-fleshed apple snack intake. In urine samples, data expressed as μmol as mean values ± standard error of mean (*n*=10), except for anthocyanin derivatives (nmol/h).

Table 1. SRM conditions used for quantification and metabolites detected in plasma and urine after acute intake of red-fleshed apple snack.

	Phenolic metabolites	SRM Quantification	Cone voltage (V) / Collision energy (eV)	Detected in[a]
	Catechol and pyrogallol derivatives			
1	Catechol sulphate	189 > 109	20 / 15	P (5), U (8)
2	Methyl catechol sulphate	203 > 123	20 / 15	P (5) U (7)
3	Methyl catechol glucuronide	299 > 123	40 / 15	U (6)
4	Pyrogallol sulphate or phloroglucinol sulphate	205 > 125	40 / 15	P (7) U (5)
	Benzoic acid derivatives			
5	Hydroxybenzoic acid	137 > 93	30 / 15	U (3)
6	Hydroxybenzoic acid sulphate	217 > 137	35 / 15	P (3), U (3)
7	Hydroxyhippuric acid	194 > 100	40 / 10	P (8)
8	Protocatechuic acid sulphate	233 > 153	35 / 15	P (4), U (5)
9	Vanillic acid sulphate	247 > 167	30 / 25	U (5)
10	Vanillic acid glucuronide	343 > 167	30 / 25	P (9) U (3)
	Phenylacetic acid derivatives			
11	Hydroxyphenylacetic acid	151 > 107	20 / 10	P (5), U (4)
12	Hydroxyphenylacetic acid sulphate	231 > 151	20 / 15	P (6), U (4)
13	Hydroxyphenylacetic acid glucuronide	327 > 151	20 / 15	U (2)
14	Dihydroxyphenylacetic acid sulphate	247 > 167	30 / 15	U (5)
15	Dihydroxyphenylacetic acid glucuronide	343 > 167	30 / 15	P (3), U (4)
	Phenylpropionic acid derivatives			
16	Hydroxyphenylpropionic acid	165 > 121	20 / 10	P (4), U (6)
17	Hydroxyphenylpropionic acid sulphate	245 > 165	35 / 15	U (7)
18	Hydroxyphenylpropionic acid glucuronide	341 > 165	40 / 25	U (4)
19	Dihydroxyphenylpropionic acid sulphate	261 > 181	40 / 15	P (2), U (5)
20	Hydroxymethoxyphenylpropionic acid	195 > 136	30 / 15	P (5), U (5)
21	Hydroxymethoxyphenylpropionic acid sulphate	275 > 195	35 / 15	P (2) U (2)
	Hydroxycinnamic acid derivatives			
22	Coumaric acid	163 > 119	35 / 10	P (2), U (5)
23	Coumaric acid sulphate	243 > 163	35 / 15	U (9)
24	Caffeic acid sulphate	259 > 179	35 / 15	P (2), U (2)
25	Ferulic acid sulphate	273 > 193	35 / 15	P (7), U (10)
	Phenyl-γ-valerolactone derivatives			
26	Hydroxyphenyl-γ-valerolactone sulphate	271 > 191	40 / 20	U (8)
27	Dihydroxyphenyl-γ-valerolactone	207 > 163	40 / 15	U (8)
28	Dihydroxyphenyl-γ-valerolactone glucuronide	383 > 207	40 / 20	U (8)
29	Dihydroxyphenyl-γ-valerolactone sulphate glucuronide	463 > 287	40 / 20	U (7)
	Flavan-3-ol derivatives			
30	Epicatechin sulphate	369 > 289	40 / 20	U (6)
31	Epicatechin glucuronide	465 > 289	40 / 20	U (6)
32	Methyl epicatechin glucuronide	383 > 303	40 / 15	U (7)
	Dihydrochalcone derivatives			
33	Phloretin glucuronide	449 > 273	40 / 20	P (10), U (10)
34	Phloretin sulphate glucuronide	529 > 353	40 / 20	U (8)
	Anthocyanin derivatives			
35	Cyanidin-3-*O*-galactoside	449 > 287	40 / 20	P (7), U (10)
36	Cyanidin arabinoside	419 > 287	40 / 20	P (1). U (10)
37	Peonidin-3-*O*-galactoside	463 > 301	40 / 20	U (10)

[a]Metabolites detected in urine (U) and/or plasma (P). Figures in parenthesis indicate the number of samples (volunteers) in which the metabolite was detected.

SUPPORTING MATERIAL

In vivo biotransformation of (poly)phenols and anthocyanins of red-fleshed apple and identification of intake biomarkers

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- Includes Supplementary Information on nutritional composition (fiber and macronutrients) of the red-fleshed apple snack (**Table S1**); concentration of the main phenolic compounds in red fleshed apple snack. Data are expressed as mg/80 g portion dry weight (mean ± SD, *n*=3) (**Table S2**); total amounts of phenolic compounds excreted in urine (**Table S3**); as well concentrations of phenolic compounds detected in plasma (**Table S4**) after red-fleshed apple snack intake. MS spectrum of the phenolic compounds and generated metabolites after the acute intake of the red-fleshed apple snack. Collision energy applied was: a) 5 eV, b) 10 eV, c) 15 eV, d) 20 eV, and e) 25 eV (**Figure S1**).

- **MATERIAL AND METHODS**

  - **Nutritional facts.** The samples were analysed for moisture, fat, protein, fibre (soluble and insoluble), sugars (reducing and non-reducing sugars) and ash content. All reagents used were of analytical grade. Moisture was estimated by weight difference after drying. Total protein content was estimated by the Dumas method and total fat was extracted with hexane from previously dried samples using a Soxhlet extractor. Insoluble fibre was determined following the method described by Van Soest et al. (1991), while soluble fibre was calculated gravimetrically as the alcohol insoluble residue according to Maran (2015). Sugar quantification (reducing and non-reducing sugars) was carried out by titrimetry based on the Fehling reaction in alkaline media after acid hydrolysis of non-reducing sugars. Ash content was determined by incineration at 550°C ± 10°C. Finally, total carbohydrates were calculated by difference. Results were expressed as grams of each compound per 100 grams of sample (g/100 g).

- **REFERENCES**

- Van Soest, P.J., Robertson, J.B., Lewis, B.A. (1991). Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *Journal of Dietary Sciences*, 74, 3583-3597. https://doi.org/10.3168/jds.S0022-0302(91)78551-2
- Maran, J.P. (2015). Statistical optimization of aqueous extraction of pectin from waste durian rinds. *International Journal of Biological Macromolecules*, 73, 92-98. https://doi.org/10.1016/j.ijbiomac.2014.10.050

Table S1. Nutritional facts of red-fleshed apple snack

Nutritional composition	Per 100g	Per portion (80g)
Calories (KJ)	982.6	786.1
(Kcal)	234.8	187.9
Fat (g)	0.9	0.7
Total Carbohydrates (g)	93.6	74.9
- of which Sugars (g)	53.8	43.0
Protein (g)	3.0	2.4 g
Fibre (total) (g)	16.5	13.2
- Soluble fibre (g)	7.6	6.1
- Insoluble fibre (g)	8.9	7.1
Minerals (g)	1.7	1.3

Table S2. Concentration of the main phenolic compounds in red fleshed apple snack. Data are expressed as mg/80 g portion dry weight (mean ± SD, n=3)

Phenolic compound	Concentration (mg/80 g portion)	
Anthocyanins		*42.3 ± 1.18*
Cyanidin-3-*O*-galactoside	39.7 ± 1.03	
Cyanidin arabinoside	2.60 ± 0.24	
Phenoloc acids		*88.0 ± 3.34*
Protocatechuic acid	1.71 ± 1.06	
Coumaric acid hexoside	0.77 ± 0.11	
Ferulic acid hexoside	2.12 ± 0.26	
Vanillic acid hexoside	4.28 ± 0.11	
Chlorogenic acid	79.1 ± 2.75	
Flavan-3-ols		*13.8 ± 1.18*
Epicatechin	5.58 ± 0.78	
Dimer	6.92 ± 0.29	
Trimer	1.30 ± 0.12	
Flavonol		*17.3 ± 1.97*
Quercetin-3-*O*-arabinoside	3.67 ± 0.45	
Quercetin-3-*O*-rhamnoside	9.26 ± 0.94	
Quercetin-3-*O*-glucoside	4.41 ± 0.57	
Flavanone		*0.42 ± 0.02*
Eriodictyol	0.42 ± 0.02	
Dihydrochalcones		*33.7 ± 3.08*
Phloretin glucoside	21.7 ± 2.54	
Phloretin xylosyl glucoside	11.7 ± 0.52	
Hydroxyphloretin xylosyl glucoside	0.32 ± 0.03	
TOTAL Phenols		*196 ± 10.7*

Table S3. Urinary excretion of phenolic compounds 0–24 h after acute intake of red-fleshed apple snack. Except for anthocyanin derivatives (nmol), data expressed in µmol as mean values ± standard deviation after subtraction of baseline excretion from each volunteer.

Phenolic metabolite[a]	Basal[b]	0-2 h	2-4 h	4-8 h
Catechol and pyrogallol derivatives				
Catechol sulphate (*n*=8)	21.7 ± 3.60	2.10 ± 0.90	1.86 ± 0.80	18.2 ± 8.4
Methyl catechol sulphate (*n*=7)	16.6 ± 4.80	0.82 ± 0.18	0.10 ± 0.05	1.64 ± 1.3
Methyl catechol glucuronide (*n*=6)	0.27 ± 0.05	0.03 ± 0.01	0.00 ± 0.00	0.03 ± 0.0
Pyrogallol sulphate or phloroglucinol sulphate (*n*=5)	1.53 ± 0.40	0.05 ± 0.03	0.15 ± 0.07	1.20 ± 0.8
Benzoic acid derivatives				
Hydroxybenzoic acid (*n*=3)	35.3 ± 10.3	18.3 ± 12.5	16.1 ± 8.01	15.4 ± 12.3
Hydroxybenzoic acid sulphate (*n*=3)	125 ± 38.1	16.2 ± 9.17	8.17 ± 3.05	2.15 ± 2.1
Protocatechuic acid sulphate (*n*=5)	16.0 ± 3.74	2.85 ± 0.88	11.5 ± 2.79	4.23 ± 1.8
Vanillic acid sulphate (*n*=5)	36.3 ± 7.15	3.51 ± 1.74	14.5 ± 8.71	28.9 ± 28.9
Vanillic acid glucuronide (*n*=3)	2.12 ± 0.74	0.15 ± 0.11	0.53 ± 0.15	1.65 ± 1.3
Phenylacetic acid derivatives				
Hydroxyphenylacetic acid (*n*=4)	210 ± 26.3	8.59 ± 3.87	14.5 ± 5.59	73.5 ± 34.4
Hydroxyphenylacetic acid sulphate (*n*=4)	52.0 ± 6.22	11.7 ± 5.32	6.20 ± 2.14	6.34 ± 3.6
Hydroxyphenylacetic acid glucuronide (*n*=2)	2.03 ± 2.03	6.92 ± 0.66	3.69 ± 1.34	2.14 ± 0.1
Dihydroxyphenylacetic acid sulphate (*n*=5)	24.6 ± 4.27	3.52 ± 1.30	2.01 ± 0.89	9.07 ± 4.1
Dihydroxyphenylacetic acid glucuronide (*n*=4)	4.72 ± 0.26	0.90 ± 0.43	4.77 ± 2.62	5.37 ± 4.1
Phenylpropionic acid derivatives				
Hydroxyphenylpropionic acid (*n*=6)	2.60 ± 0.88	1.86 ± 0.90	2.65 ± 0.98	3.79 ± 1.6
Hydroxyphenylpropionic acid sulphate (*n*=7)	36.6 ± 11.1	4.35 ± 0.96	2.34 ± 0.52	13.4 ± 5.6
Hydroxyphenylpropionic acid glucuronide (*n*=4)	1.57 ± 0.70	0.62 ± 0.41	0.13 ± 0.06	0.22 ± 0.0
Dihydroxyphenylpropionic acid sulphate (*n*=5)	21.6 ± 2.83	2.86 ± 2.08	3.26 ± 1.61	22.4 ± 11.9
Hydroxymethoxyphenylpropionic acid (*n*=5)	0.15 ± 0.04	0.02 ± 0.01	0.09 ± 0.04	0.29 ± 0.1
Hydroxymethoxyphenylpropionic acid sulphate (*n*=2)	0.41 ± 0.16	0.09 ± 0.04	0.09 ± 0.01	0.48 ± 0.1
Hydroxycinnamic acid derivatives				
Coumaric acid (*n*=5)	0.07 ± 0.05	0.32 ± 0.08	0.64 ± 0.12	1.28 ± 0.3
Coumaric acid sulphate (*n*=9)	0.77 ± 0.26	0.49 ± 0.10	0.80 ± 0.17	1.48 ± 0.3
Caffeic acid sulphate (*n*=2)	1.31 ± 0.91	0.51 ± 0.32	1.00 ± 0.74	1.30 ± 1.1
Ferulic acid sulphate (*n*=10)	1.43 ± 0.41	0.29 ± 0.08	0.59 ± 0.11	0.74 ± 0.2
Phenyl-γ-valerolactone derivatives				
Hydroxylphenyl-γ-valerolactone sulphate (*n*=8)	0.15 ± 0.15	0.02 ± 0.01	0.04 ± 0.02	1.21 ± 0.9
Dihydroxylphenyl-γ-valerolactone (*n*=8)	3.51 ± 2.25	0.98 ± 0.63	2.96 ± 1.91	15.9 ± 4.6
Dihydroxylphenyl-γ-valerolactone glucuronide (*n*=8)	0.01 ± 0.01	0.00 ± 0.00	0.01 ± 0.01	0.17 ± 0.0
Dihydroxylphenyl-γ-valerolactone sulphate glucuronide (*n*=7)	0.07 ± 0.07	0.06 ± 0.03	0.16 ± 0.08	1.34 ± 0.4
Flavan-3-ol derivatives				
Epicatechin sulphate (*n*=6)	0.14 ± 0.14	0.40 ± 0.10	0.71 ± 0.11	0.52 ± 0.1
Epicatechin glucuronide (*n*=6)	n.d.	0.24 ± 0.07	0.37 ± 0.06	0.51 ± 0.2
Methyl epicatechin glucuronide (*n*=7)	0.07 ± 0.07	0.23 ± 0.07	0.42 ± 0.08	0.36 ± 0.1
Dihydrochalcone derivatives				
Phloretin glucuronide (*n*=10)	n.d.	0.71 ± 0.10	1.26 ± 0.32	1.06 ± 0.2
Phloretin sulphate glucuronide (*n*=8)	0.03 ± 0.01	0.12 ± 0.03	0.11 ± 0.03	0.09 ± 0.0
Anthocyanin derivatives				
Cyanidin-3-*O*-galactoside (*n*=10)	n.d.	2.49 ± 0.42	3.46 ± 0.63	2.88 ± 0.5
Cyanidin arabinoside (*n*=10)	0.45 ± 0.07	0.74 ± 0.16	0.35 ± 0.11	0.35 ± 0.1
Peonidin-3-*O*-galactoside (*n*=10)	n.d.	0.82 ± 0.15	1.61 ± 0.25	1.22 ± 0.2

[a] Figures in parenthesis next to compound names indicate the number of samples (volunteers) in which the metabolite was detected.
[b] Content of urine collected for 12 h prior to supplementation and on an excretion per hour basis used to subtract from excretion values obtained after red-fleshed apple snack consumption to obtain the values cited in the Table.
n.d.: not detected

Table S4. Phenolic compounds concentrations in plasma 0–24 h after acute intake of red-fleshed apple snack. Data expressed in nmol/L as mean values ± standard deviation

Phenolic compound [a]	Basal (0 h)	0.5 h	1 h	2 h	4 h	6 h	24 h
Catechol and pyrogallol derivatives							
Catechol sulphate (*n*=5)	310 ± 59.2	367 ± 77.7	329 ± 62.2	389 ± 84.7	743 ± 243	1220 ± 434	373 ± 70.8
Methyl catechol sulphate (*n*=5)	133 ± 67.6	167 ± 79.3	195 ± 1075	159 ± 93.0	151 ± 64.5	260 ± 124	243 ± 71.3
Pyrogallol sulphate (*n*=7)	17.4 ±6.19	11.8 ± 4.84	11.6 ± 5.12	11.5 ± 5.26	17.1 ± 8.28	67.6 ± 21.0	16.3 ± 5.67
Benzoic acid derivative							
Hydroxybenzoic acid sulphate (*n*=3)	133 ± 44.1	179 ± 37.0	212 ± 52.4	132 ± 20.1	110 ± 21.3	144 ± 38.3	146 ± 52.5
Hydroxyhippuric acid (*n*=8)	13.5 ± 3.50	15.9 ± 5.68	21.3 ± 5.83	26.7 ± 7.57	27.8 ± 4.84	29.4 ± 7.33	15.8 ± 7.00
Protocatechuic acid sulphate (*n*=4)	n.d.	19.5 ± 11.5	39.3 ± 3.15	29.2 ± 4.46	20.3 ± 10.7	16.4 ± 16.4	n.d.
Vanillic acid glucuronide (*n*=9)	6.18 ± 2.52	10.7 ± 3.10	16.1 ± 2.61	23.6 ± 4.17	27.3 ± 3.25	23.9 ± 3.41	10.1 ± 2.54
Phenylacetic acid derivatives							
Hydroxyphenylacetic acid (*n*=5)	2178 ± 148	1946 ± 131	2049 ± 133	2577 ± 262	2788 ± 137	2993 ± 195	1820 ± 157
Hydroxyphenylacetic acid sulphate (*n*=6)	1016 ± 711	2047 ± 1120	2169 ± 1310	1398 ± 857	1607 ± 1166	1946 ± 1453	1523 ± 918
Dihydroxyphenylacetic acid glucuronide (*n*=3)	13.0 ± 13.0	23.3 ± 23.3	39.1 ± 20.3	63.2 ± 44.5	86.1 ± 17.6	84.6 ± 31.3	13.2 ± 13.2
Phenylpropionic acid derivatives							
Hydroxyphenylpropionic acid (*n*=4)	48.3 ± 48.3	55.4 ± 55.4	69.8 ± 69.8	265 ± 134	357 ± 186	897 ± 393	852 ± 432
Dihydroxyphenylpropionic acid sulphate (*n*=2)	n.d.	8.52 ± 0.95	9.94 ± 1.10	18.3 ± 2.04	38.1 ± 4.23	30.4 ± 3.37	10.4 ± 1.16
Hydroxymethoxyphenylpropionic acid (*n*=5)	11.1 ± 3.58	12.9 ± 2.71	12.8 ± 2.17	25.5 ± 9.89	41.6 ± 8.77	40.5 ± 8.01	37.9 ± 17.3
Hydroxymethoxyphenylpropionic acid sulphate (*n*=2)	n.d.	1.26 ± 1.26	0.99 ± 0.99	6.67 ± 0.98	11.2 ± 1.07	3.20 ± 3.20	2.87 ± 2.87
Hydroxycinnamic acid derivatives							
Coumaric acid (*n*=2)	143 ± 10.8	148 ± 24.8	171 ± 0.47	194 ± 24.2	165 ± 18.6	292 ± 30.5	157 ± 16.4
Caffeic acid sulphate (*n*=2)	8.21 ± 1.99	29.1 ± 14.3	37.5 ± 13.8	14.3 ± 8.06	11.8 ± 4.59	18.7 ± 4.78	13.0 ± 1.05
Ferulic acid sulphate (*n*=7)	18.2 ± 4.57	25.9 ± 4.76	25.9 ± 3.64	20.6 ± 3.09	24.8 ± 6.01	30.0 ± 3.45	17.7 ± 2.82
Dihydrochalcone derivatives							
Phloretin sulphate (*n*=2)	n.d.	20.7 ± 16.3	18.4 ± 12.4	8.35 ± 5.01	8.43 ± 5.00	24.0 ± 4.18	10.9 ± 6.81
Phloretin glucuronide (*n*=10)	n.d.	28.1 ± 3.54	46.7 ± 1.57	61.0 ± 6.82	53.9 ± 11.0	34.5 ± 11.7	1.16 ± 1.16
Anthocyanin derivatives							
Cyanidin-3-*O*-galactoside (*n*=7)	n.d.	9.66 ± 2.26	9.15 ± 1.81	10.3 ± 1.50	6.86 ± 0.74	1.52 ± 0.82	0.30 ± 0.30
Cyanidin arabinoside (*n*=1)	n.d.	2.60	n.d.	n.d.	n.d.	n.d.	n.d.

[a]Figures in parenthesis next to compound names indicate the number of samples (volunteers) in which the metabolite was detected.
n.d.: not detected

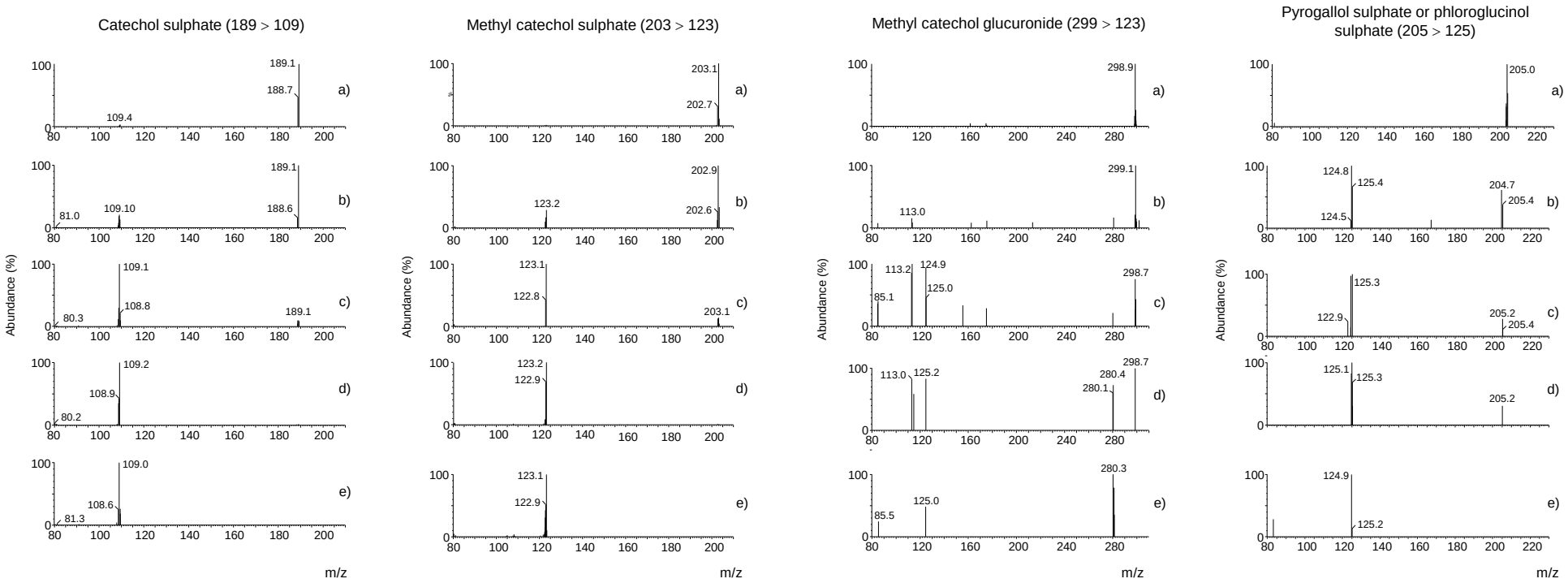
Catechol sulphate (189 > 109)
Methyl catechol sulphate (203 > 123)
Methyl catechol glucuronide (299 > 123)
Pyrogallol sulphate or phloroglucinol sulphate (205 > 125)
Abundance (%)
m/z
a)
b)
c)
d)
e)

Figure S1

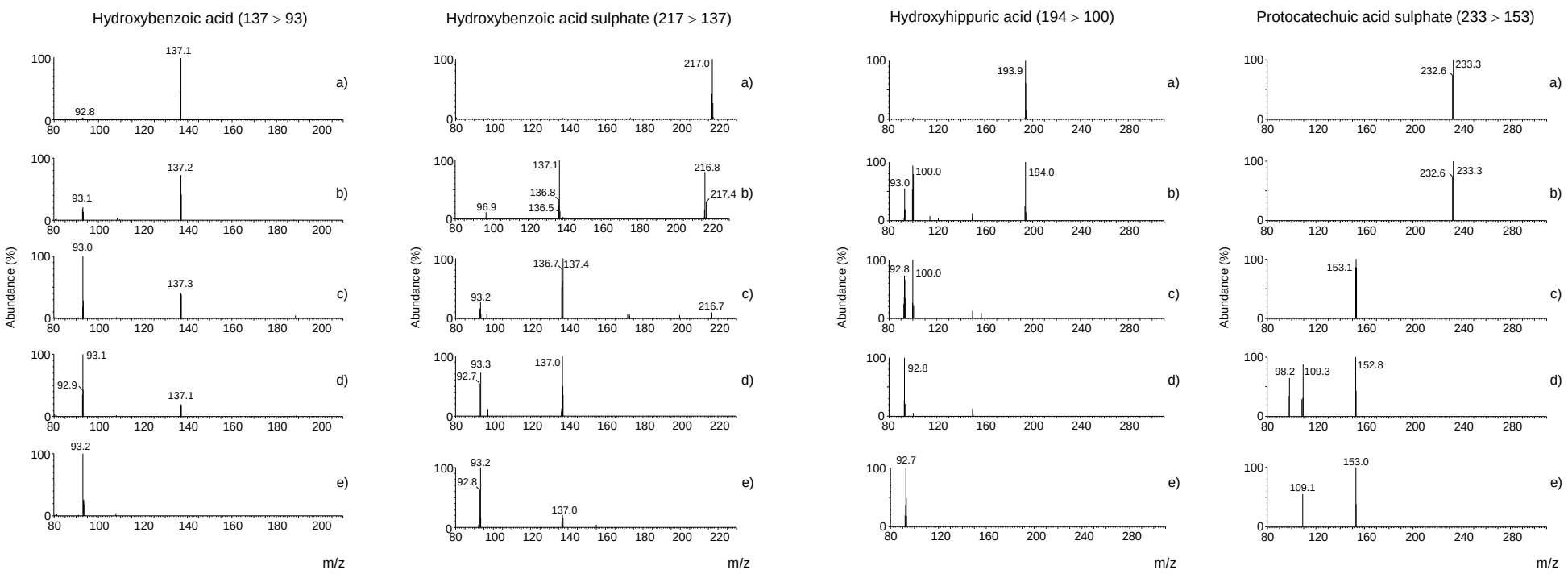
Hydroxybenzoic acid (137 > 93)
Hydroxybenzoic acid sulphate (217 > 137)
Hydroxyhippuric acid (194 > 100)
Protocatechuic acid sulphate (233 > 153)
Abundance (%)
m/z
a)
b)
c)
d)
e)
137.1
92.8
137.2
93.1
93.0
137.3
93.1
92.9
137.1
93.2
217.0
137.1
216.8
136.8
217.4
96.9
136.5
136.7
137.4
93.2
216.7
93.3
92.7
137.0
93.2
92.8
137.0
193.9
93.0
100.0
194.0
92.8
100.0
92.8
92.7
232.6
233.3
232.6
233.3
153.1
98.2
109.3
152.8
153.0
109.1

Figure S1

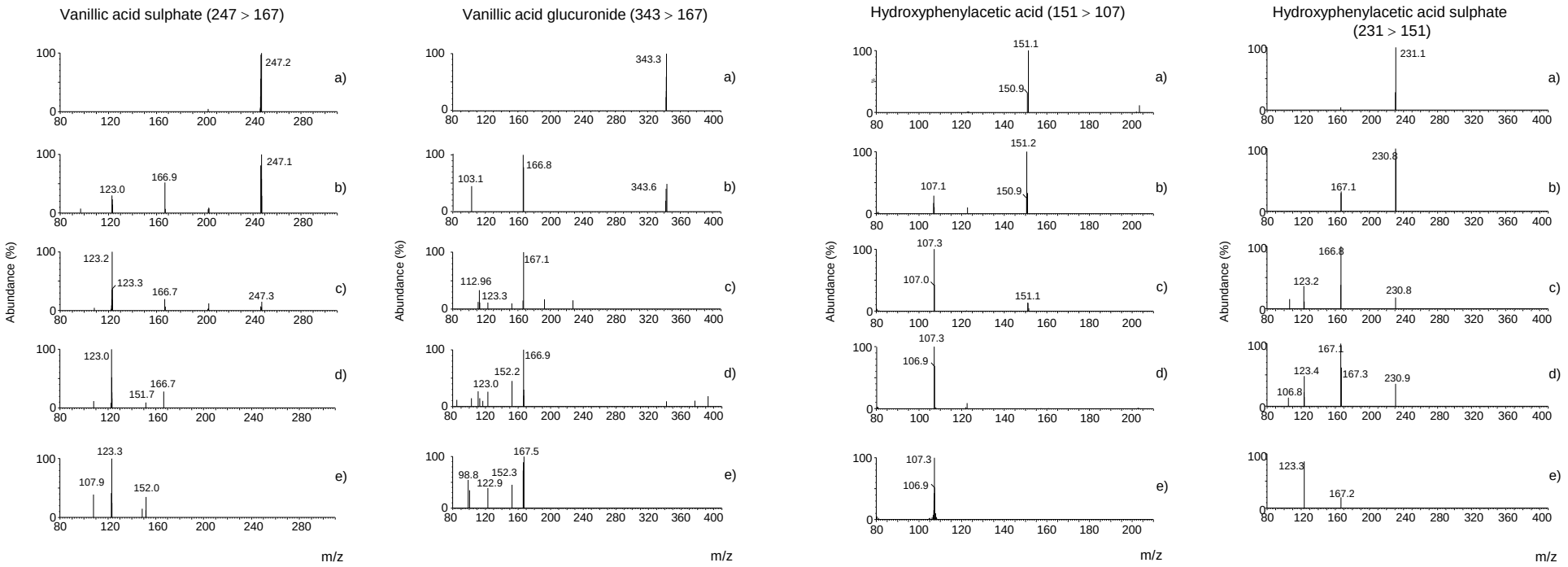
Vanillic acid sulphate (247 > 167)
Vanillic acid glucuronide (343 > 167)
Hydroxyphenylacetic acid (151 > 107)
Hydroxyphenylacetic acid sulphate (231 > 151)
Abundance (%)
m/z
a)
b)
c)
d)
e)
247.2
247.1
123.0
166.9
123.2
123.3
166.7
247.3
123.0
151.7
166.7
123.3
107.9
152.0
343.3
103.1
166.8
343.6
167.1
112.96
123.3
166.9
152.2
123.0
167.5
98.8
122.9
152.3
151.1
150.9
151.2
107.1
150.9
107.3
107.0
151.1
107.3
106.9
107.3
106.9
231.1
230.8
167.1
166.8
123.2
230.8
167.1
123.4
106.8
167.3
230.9
123.3
167.2

Figure S1

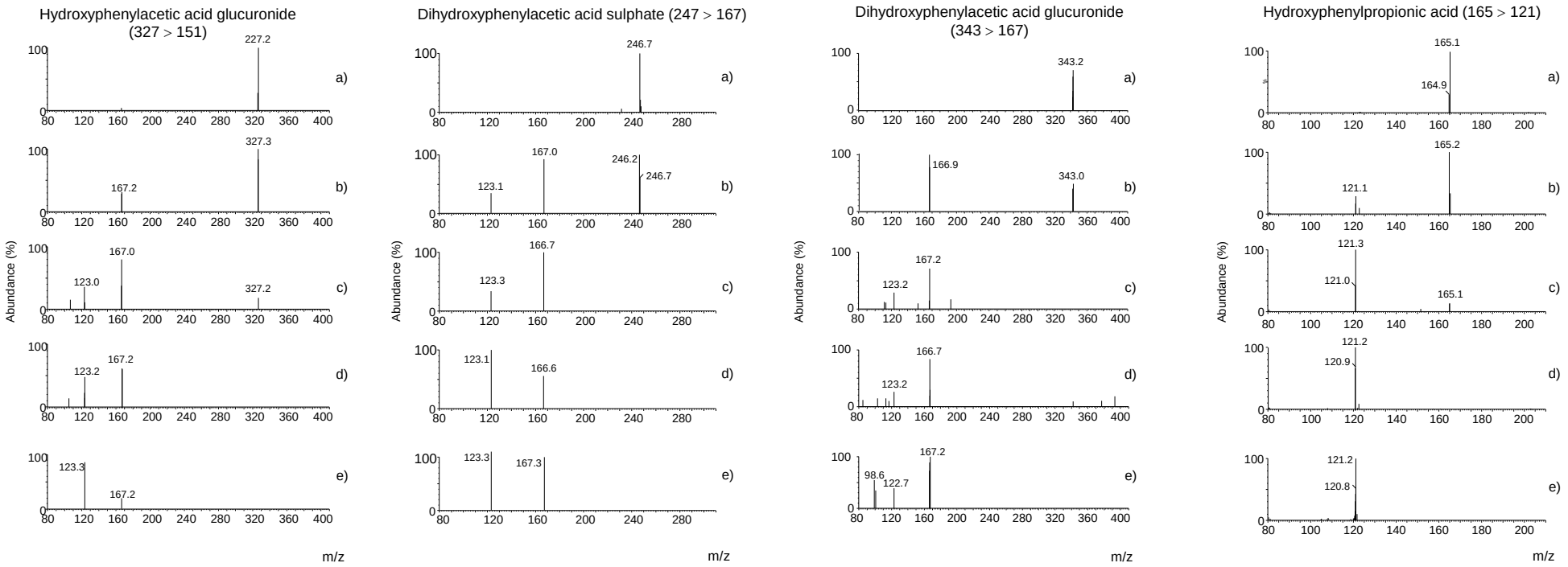
Hydroxyphenylacetic acid glucuronide
(327 > 151)
Dihydroxyphenylacetic acid sulphate (247 > 167)
Dihydroxyphenylacetic acid glucuronide
(343 > 167)
Hydroxyphenylpropionic acid (165 > 121)
Abundance (%)
m/z
a)
b)
c)
d)
e)
227.2
327.3
167.2
167.0
123.0
327.2
123.2
123.3
246.7
123.1
246.2
166.7
166.6
167.3
343.2
166.9
343.0
98.6
122.7
165.1
164.9
165.2
121.1
121.3
121.0
121.2
120.9
120.8

Figure S1

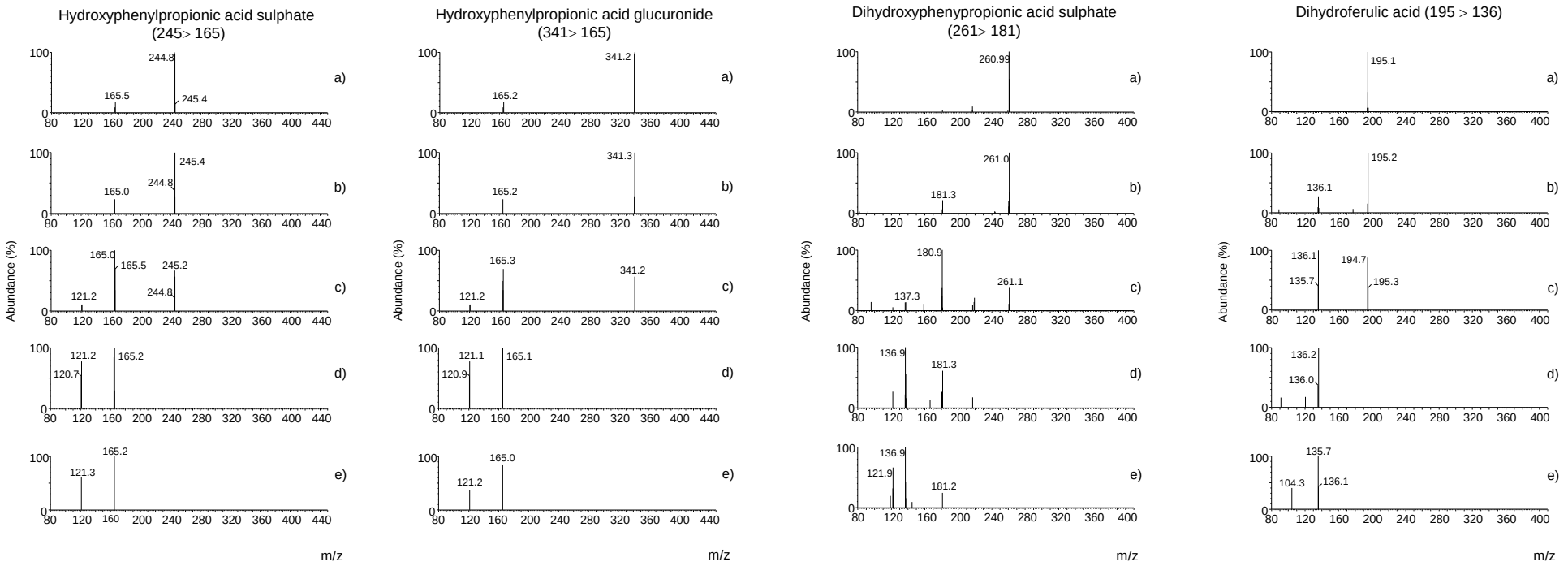
Hydroxyphenylpropionic acid sulphate
(245> 165)
Hydroxyphenylpropionic acid glucuronide
(341> 165)
Dihydroxyphenypropionic acid sulphate
(261> 181)
Dihydroferulic acid (195 > 136)
Abundance (%)
m/z
a)
b)
c)
d)
e)
244.8
165.5
245.4
165.0
245.4
244.8
165.0
165.5
245.2
244.8
121.2
121.2
120.7
165.2
121.3
165.2
341.2
165.2
341.3
165.2
165.3
341.2
121.2
121.1
120.9
165.1
165.0
121.2
260.99
261.0
181.3
180.9
137.3
261.1
136.9
181.3
136.9
121.9
181.2
195.1
195.2
136.1
136.1
135.7
194.7
195.3
136.2
136.0
135.7
104.3
136.1

Figure S1

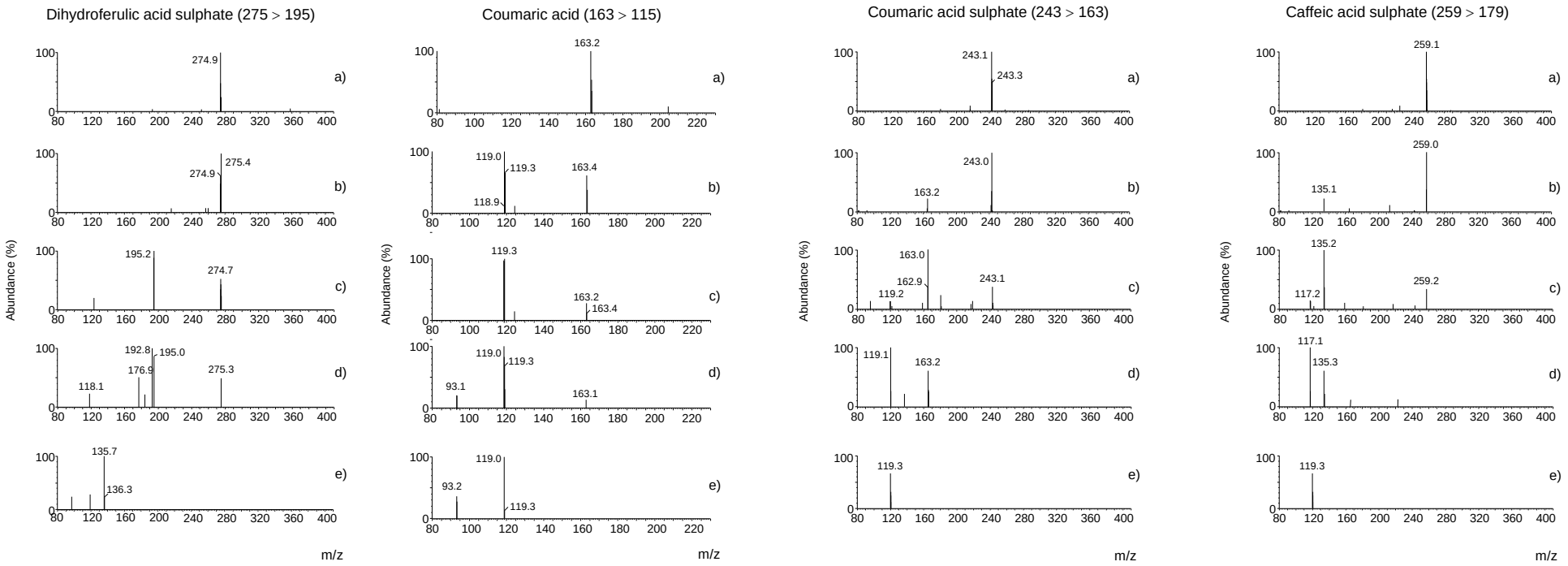
Dihydroferulic acid sulphate (275 > 195)
Coumaric acid (163 > 115)
Coumaric acid sulphate (243 > 163)
Caffeic acid sulphate (259 > 179)
Abundance (%)
m/z
a)
b)
c)
d)
e)
274.9
275.4
195.2
274.7
192.8
195.0
176.9
275.3
118.1
135.7
136.3
163.2
119.0
119.3
163.4
118.9
163.2
163.4
93.1
163.1
93.2
243.1
243.3
243.0
163.0
162.9
119.2
119.1
259.1
259.0
135.1
135.2
259.2
117.2
117.1
135.3

Figure S1

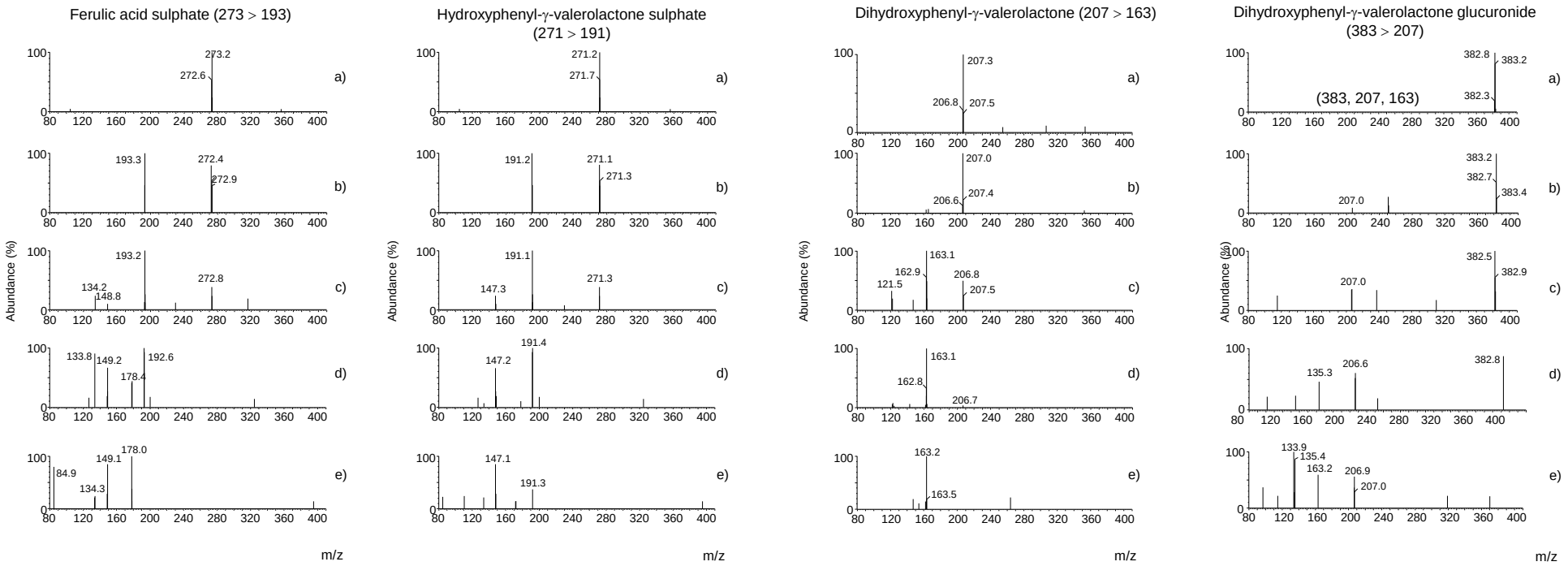
Ferulic acid sulphate (273 > 193)
Hydroxyphenyl-γ-valerolactone sulphate (271 > 191)
Dihydroxyphenyl-γ-valerolactone (207 > 163)
Dihydroxyphenyl-γ-valerolactone glucuronide (383 > 207)
Abundance (%)
m/z
a)
b)
c)
d)
e)
273.2
272.6
193.3
272.4
272.9
193.2
134.2
148.8
272.8
133.8
149.2
192.6
178.4
84.9
149.1
178.0
134.3
271.2
271.7
191.2
271.1
271.3
191.1
147.3
271.3
147.2
191.4
147.1
191.3
207.3
206.8
207.5
207.0
206.6
207.4
163.1
162.9
121.5
206.8
207.5
163.1
162.8
206.7
163.2
163.5
382.8
383.2
(383, 207, 163)
382.3
383.2
382.7
383.4
207.0
382.5
382.9
207.0
135.3
206.6
382.8
133.9
135.4
163.2
206.9
207.0

Figure S1

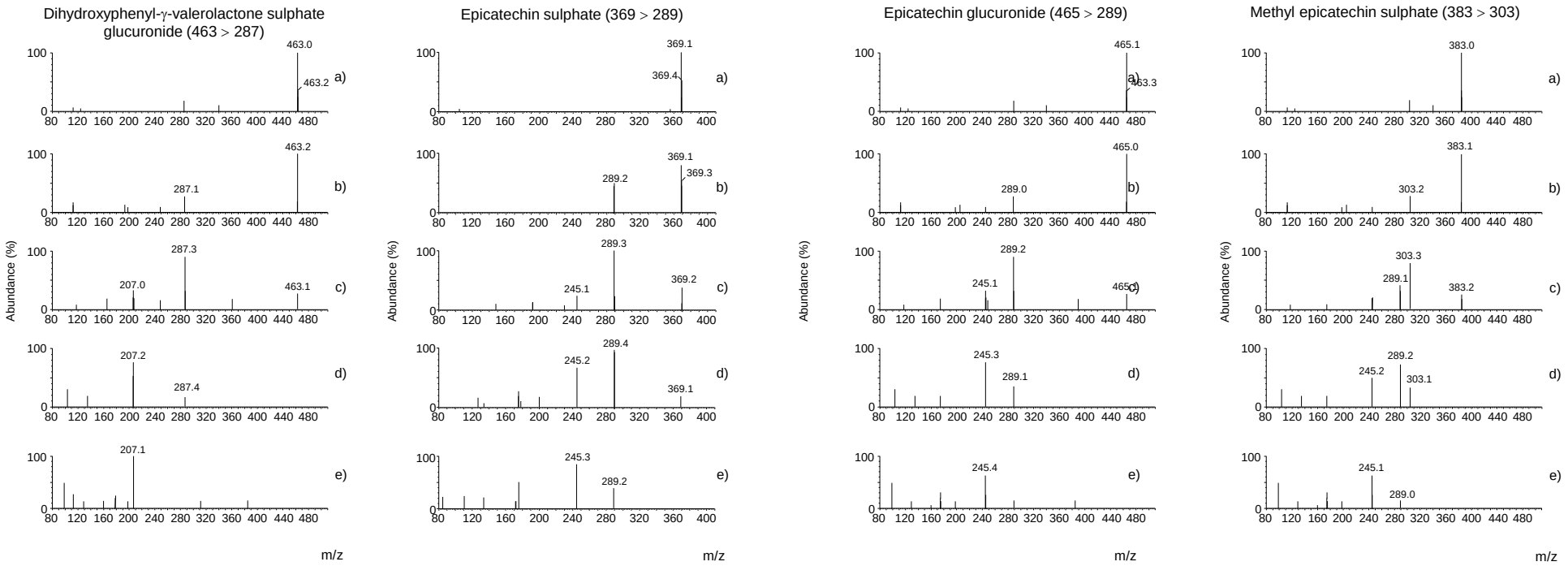
Dihydroxyphenyl-γ-valerolactone sulphate glucuronide (463 > 287)
Epicatechin sulphate (369 > 289)
Epicatechin glucuronide (465 > 289)
Methyl epicatechin sulphate (383 > 303)
Abundance (%)
m/z
a)
b)
c)
d)
e)

Figure S1

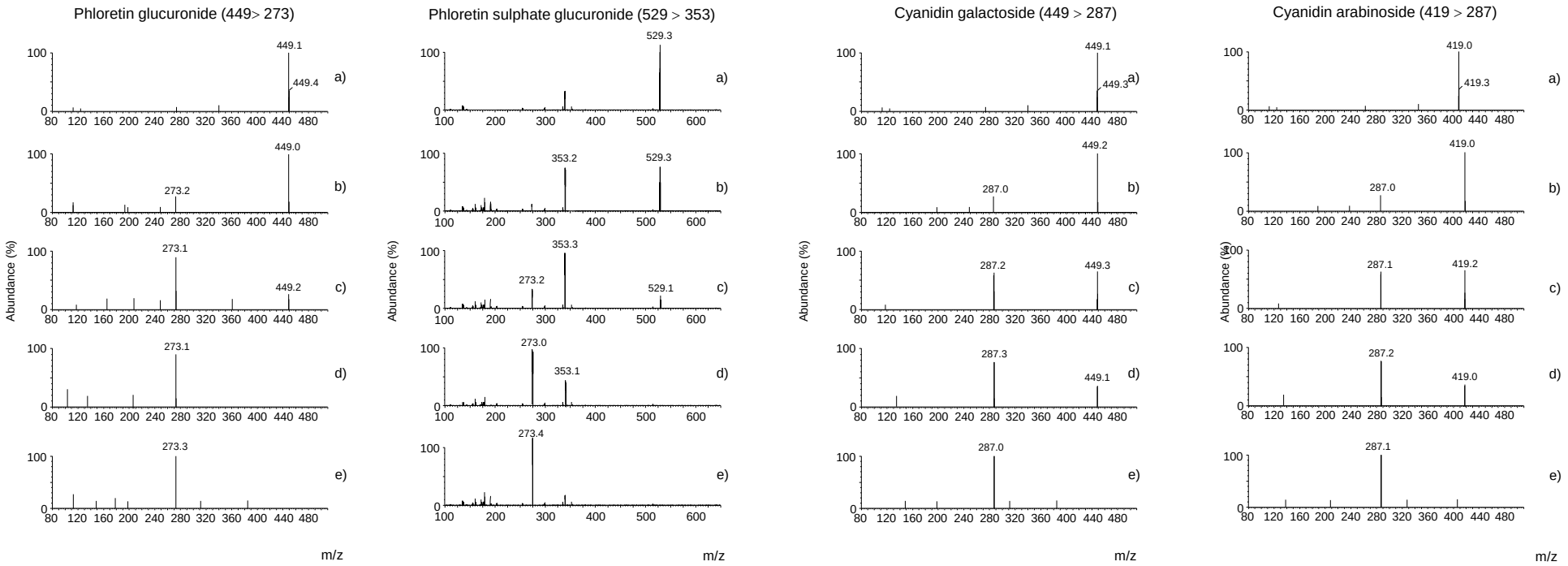
Phloretin glucuronide (449> 273)
Phloretin sulphate glucuronide (529 > 353)
Cyanidin galactoside (449 > 287)
Cyanidin arabinoside (419 > 287)
Abundance (%)
m/z
a)
b)
c)
d)
e)
449.1
449.4
449.0
273.2
273.1
449.2
273.3
529.3
353.2
529.3
353.3
273.2
529.1
273.0
353.1
273.4
449.1
449.3
449.2
287.0
287.2
449.3
287.3
449.1
287.0
419.0
419.3
419.0
287.0
287.1
419.2
287.2
419.0
287.1

Figure S1

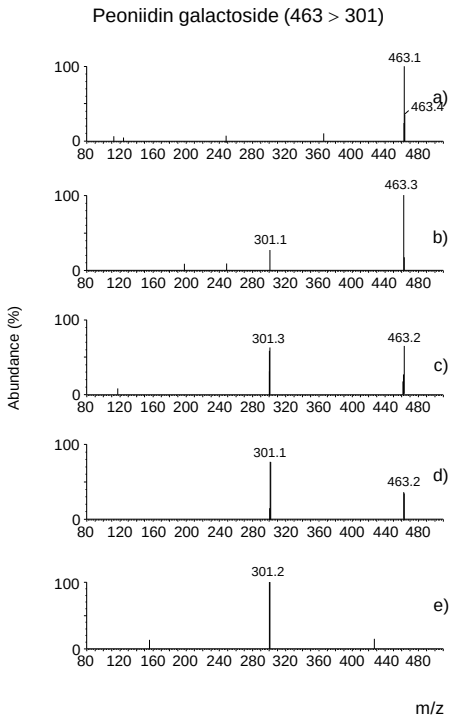
Peoniidin galactoside (463 > 301)
Abundance (%)
100
0
80 120 160 200 240 280 320 360 400 440 480
463.1
463.4
a)
463.3
301.1
b)
301.3
463.2
c)
301.1
463.2
d)
301.2
e)
m/z

Figure S1

Figure 2

Click here to download Figure: Revised_Figure 2_Yuste_et_al_JFF.ppt

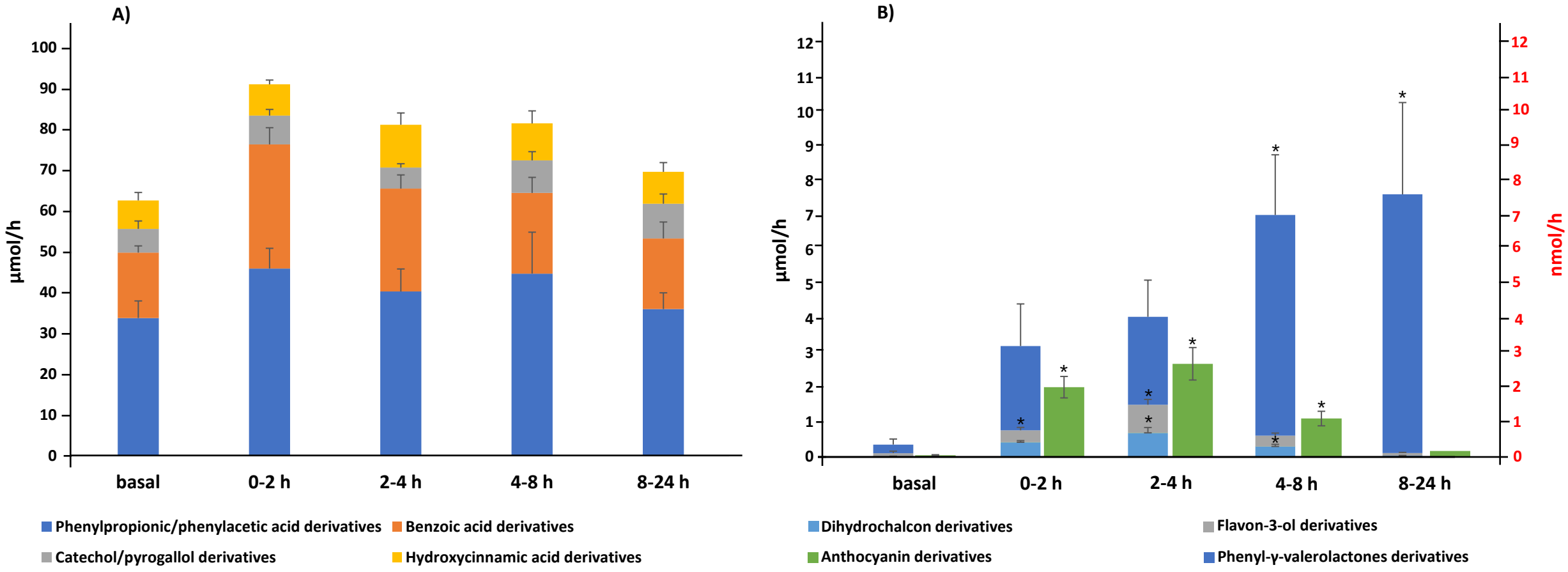

Figure 2

Figure 3

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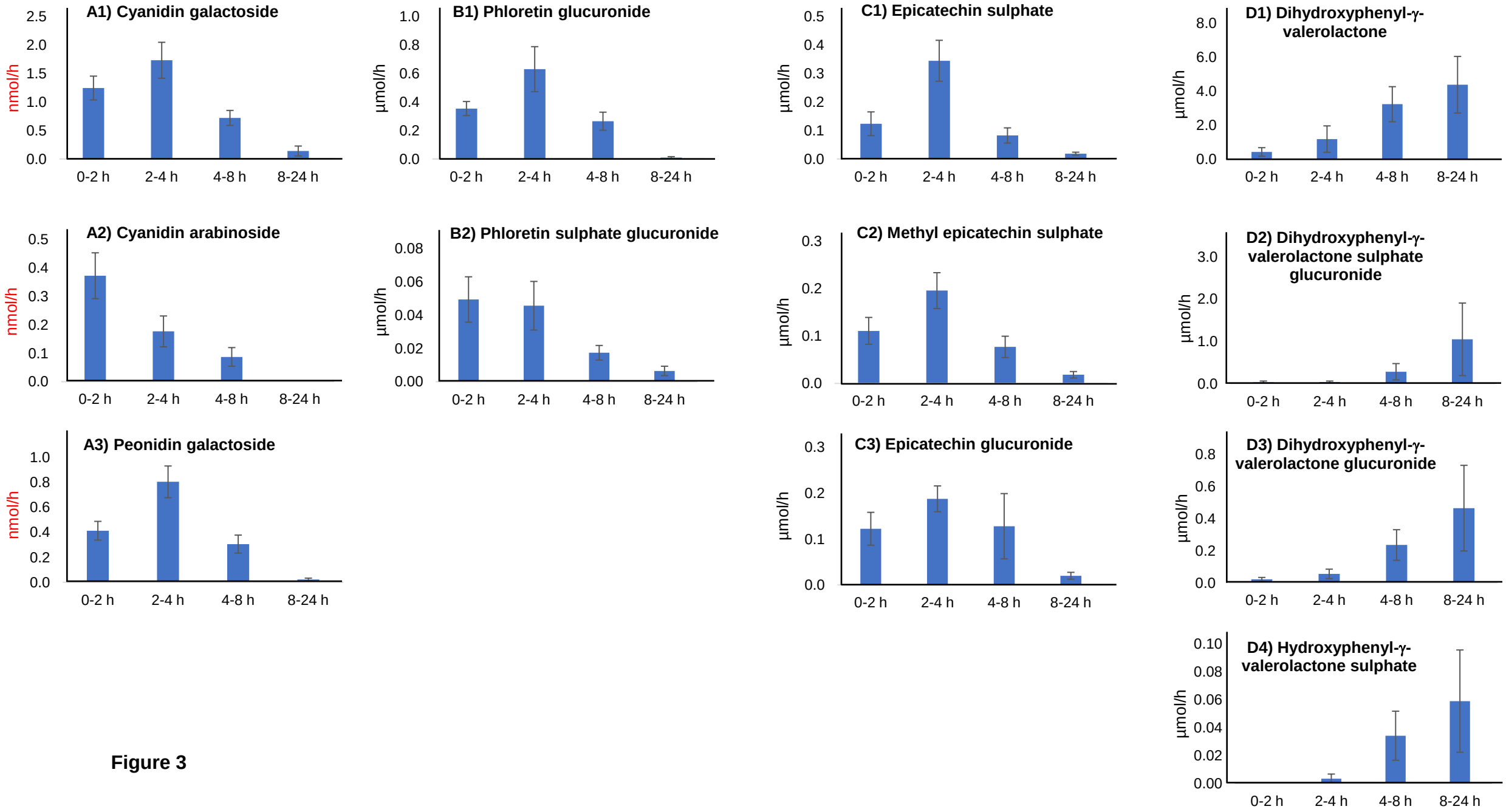


Figure 3

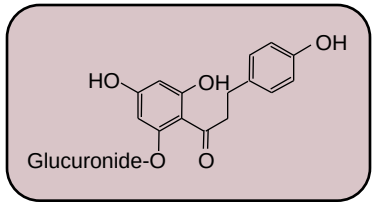
Figure 4

Click here to download Figure: Revised_Figure 4_Yuste_et_al_JFF.ppt

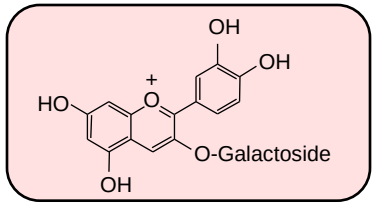
Red-fleshed Apple snack

Human Acute intake

Selected Intake biomarkers

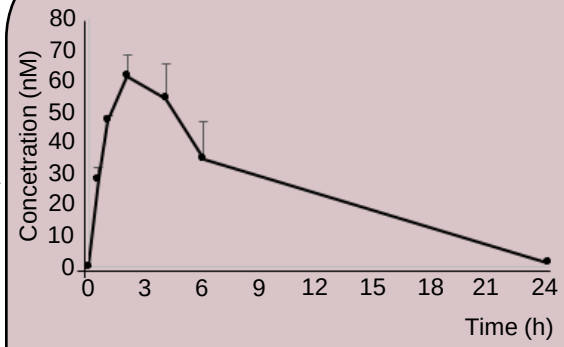


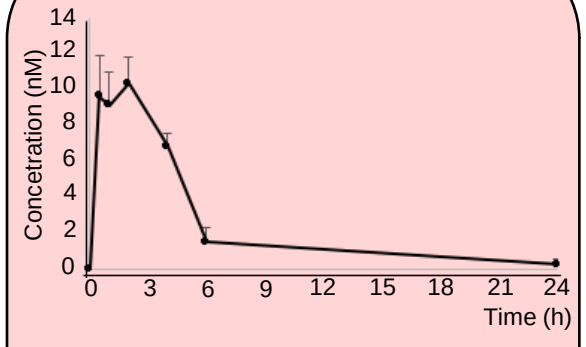
Phloretin glucuronide



Cyanidin galactoside

PLASMA





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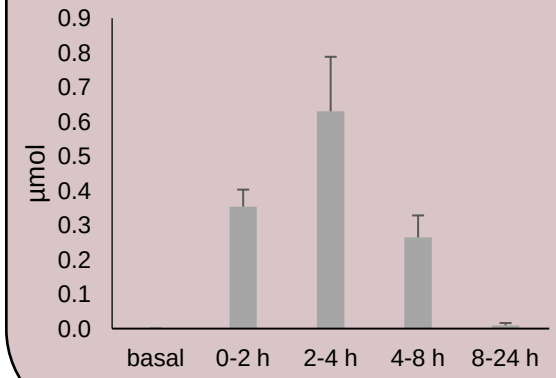

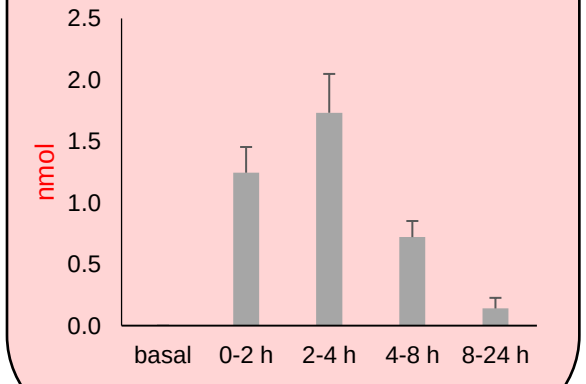

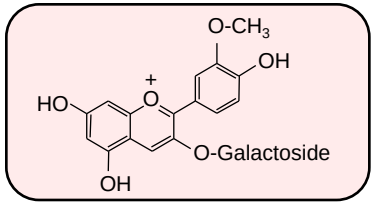

Peonidin galactoside

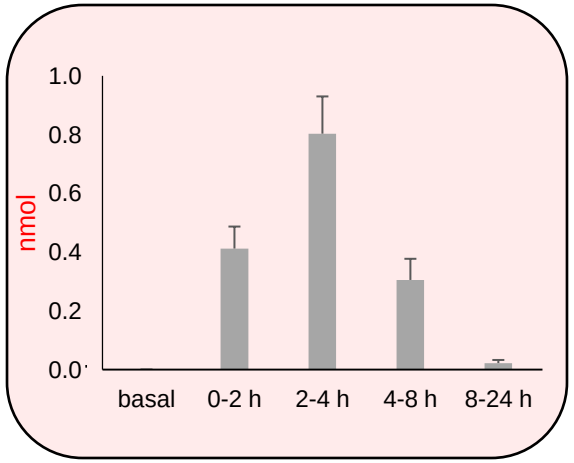

Figure 1

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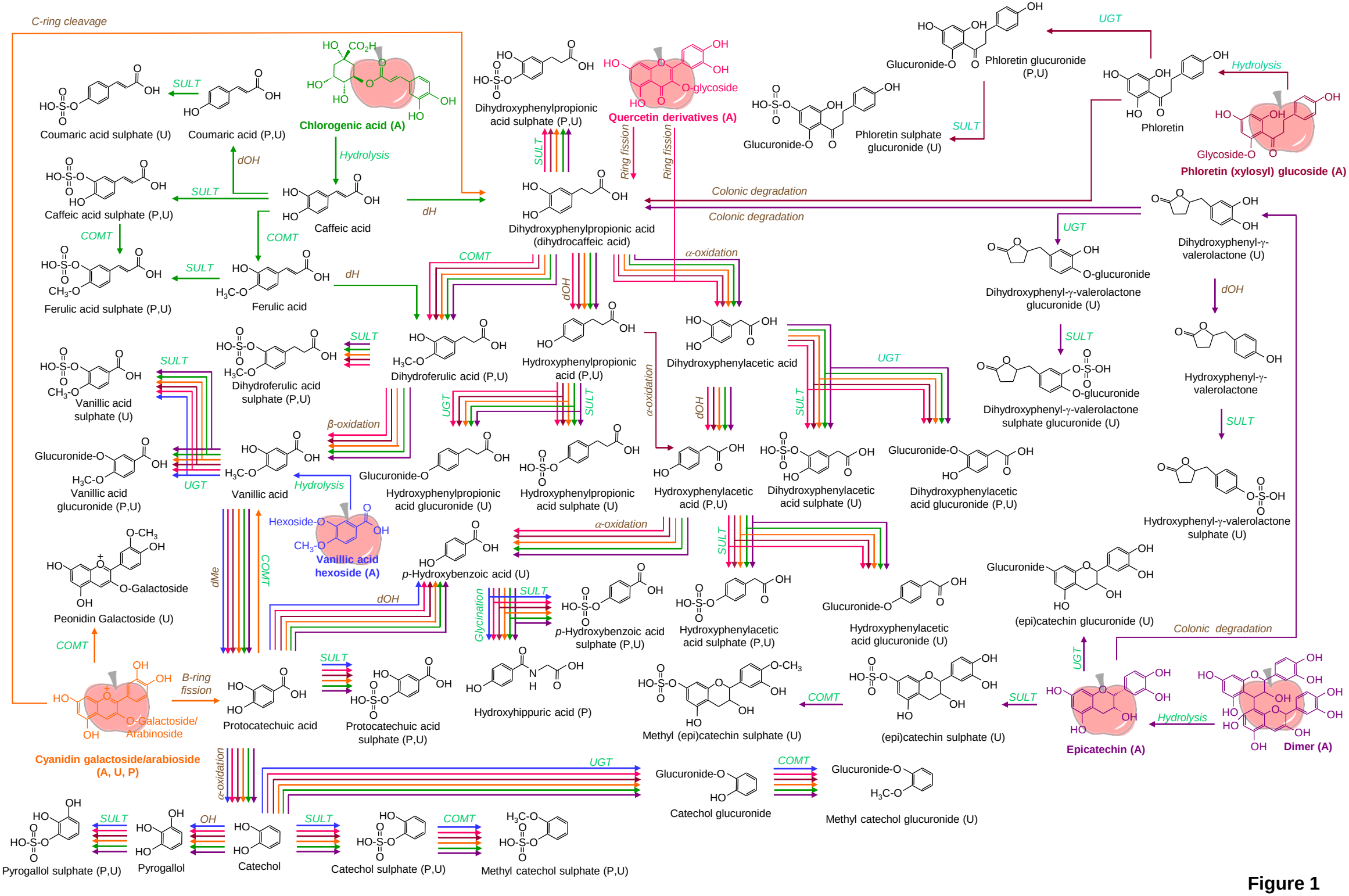


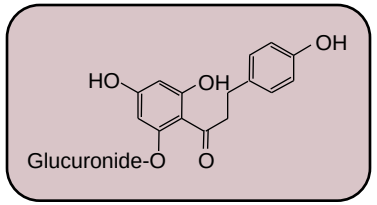
Figure 1

*Graphical Abstract

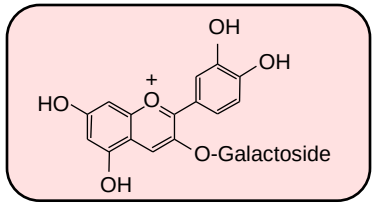
Red-fleshed Apple snack

Human Acute intake

Selected Intake biomarkers

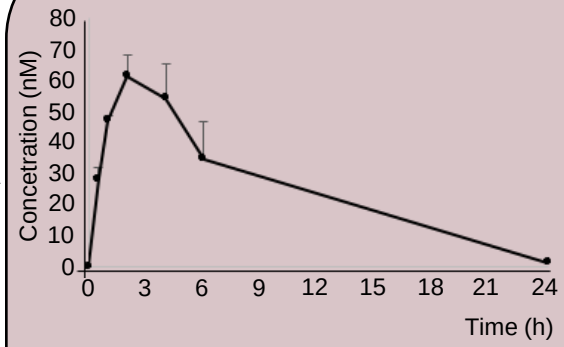


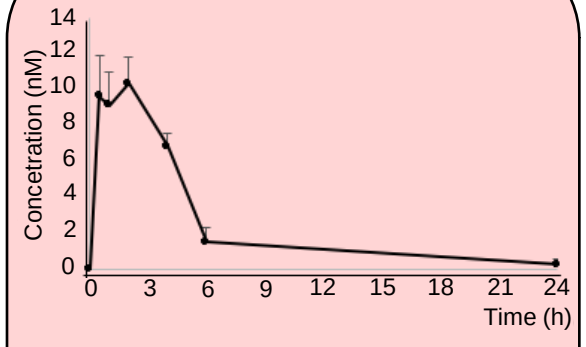
Phloretin glucuronide



Cyanidin galactoside

PLASMA





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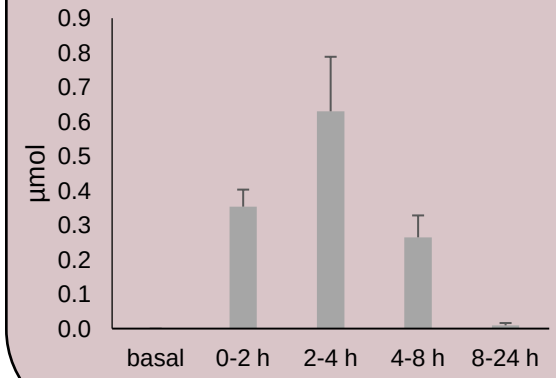
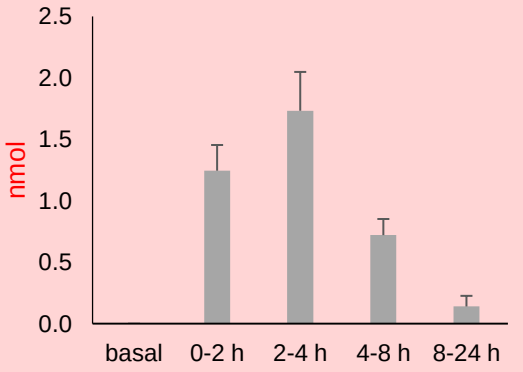
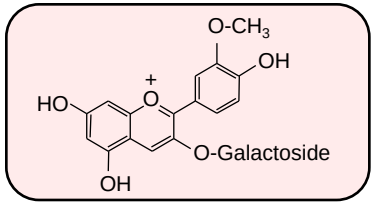
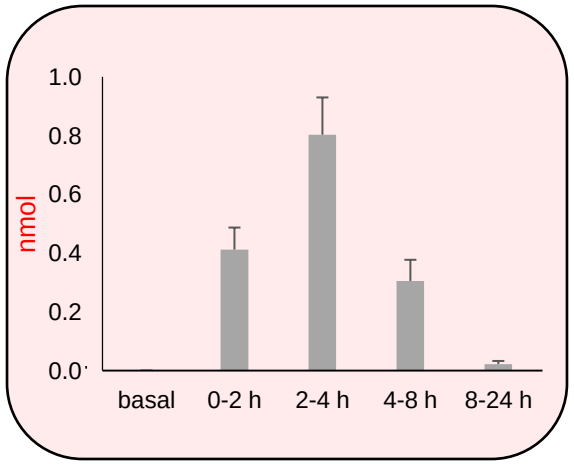






Peonidin galactoside



CONFLICT OF INTEREST

On behalf of all authors, the corresponding author states that there is no conflict of interest.

ETHICS STATEMENT

The protocol of the study was approved by the Ethical Committee of the Human Clinical Research Unit at the Arnau Vilanova University Hospital, Lleida, Spain (Approval Number: 13/2016).